

This Page Is Inserted by IFW Operations  
and is not a part of the Official Record

## **BEST AVAILABLE IMAGES**

Defective images within this document are accurate representations of the original documents submitted by the applicant.

Defects in the images may include (but are not limited to):

- BLACK BORDERS
- TEXT CUT OFF AT TOP, BOTTOM OR SIDES
- FADED TEXT
- ILLEGIBLE TEXT
- SKEWED/SLANTED IMAGES
- COLORED PHOTOS
- BLACK OR VERY BLACK AND WHITE DARK PHOTOS
- GRAY SCALE DOCUMENTS

**IMAGES ARE BEST AVAILABLE COPY.**

**As rescanning documents *will not* correct images,  
please do not report the images to the  
Image Problem Mailbox.**

**THIS PAGE BLANK (USPTO)**



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

<b>(51) International Patent Classification <sup>7</sup> :</b> <b>C12N 15/86, 7/01, 5/10, A61K 39/00, 39/145, 48/00</b>	<b>A1</b>	<b>(11) International Publication Number:</b> <b>WO 00/53786</b> <b>(43) International Publication Date:</b> 14 September 2000 (14.09.00)
<b>(21) International Application Number:</b> PCT/EP00/01903 <b>(22) International Filing Date:</b> 3 March 2000 (03.03.00) <b>(30) Priority Data:</b> 99104519.6                      6 March 1999 (06.03.99)                      EP <b>(71) Applicant (for all designated States except US):</b> ARTEMIS PHARMACEUTICALS GMBH [DE/DE]; Neurather Ring 1, D-51063 Köln (DE). <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> HOBOM, Gerd [DE/DE]; Arndtstrasse 14, D-35392 Giessen (DE). FLICK, Ramon [DE/SE]; Lilla Hallsatrvägen 28, S-137 93 Västerhaninge (SE). MENKE, Anette [DE/DE]; Ockershäuser Allee 7, D-35037 Marburg (DE). AZZEH, Maysa [IL/DE]; Altenfeldsweg 32, D-35394 Giessen (DE). <b>(74) Agents:</b> HELBING, Jörg et al.; Von Kreisler Selting Werner, Deichmannhaus am Dorn, D-50667 Köln (DE).		<b>(81) Designated States:</b> AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

**(54) Title:** STABLE RECOMBINANT INFLUENZA VIRUSES FREE OF HELPER VIRUSES**(57) Abstract**

The invention relates to a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus and which comprises at least one viral RNA segment being an ambisense RNA molecule (ambisense RNA segment) and containing one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or *vice versa*, in overall convergent arrangement. The present invention further provides a method for the production of said recombinant influenza virus, a method for constructing specific ribozyme-sensitive influenza carrier strains; pharmaceutical compositions comprising said recombinant influenza virus; and the use of said recombinant influenza virus for preparing medicaments for vaccination purposes.

ATTORNEY DOCKET NUMBER: 7682-051-999  
 SERIAL NUMBER: 09/724,388  
 REFERENCE: CJ

**FOR THE PURPOSES OF INFORMATION ONLY**

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LJL	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav	TM	Turkmenistan
BF	Burkina Faso	GR	Greece		Republic of Macedonia	TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NB	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's	NZ	New Zealand		
CM	Cameroon		Republic of Korea	PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakhstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

## Stable Recombinant Influenza Viruses Free of Helper Viruses

### 5 Field of the Invention

The invention relates to a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus; a method for the production of said recombinant influenza virus, a method for constructing specific ribozyme-sensitive influenza carrier strains; pharmaceutical compositions comprising  
10 said recombinant influenza viruses; and the use of said recombinant influenza virus for preparing medicaments for vaccination purposes.

### Technical Background

15 Redesigning influenza virus into a vector system for expression of foreign genes similar to what has been achieved in several other thoroughly studied viruses such as adenovirus, retrovirus, Semliki Forest virus or Rabies virus has the advantage of an industrially well established mode of cheap propagation for influenza in fertilized chicken eggs leading to rather  
20 high titers (above  $10^{10}$ /ml). On the other hand none of its genes may be deleted from the influenza genome according to our present knowledge, and give room for large-size foreign insertions. Only small fragments of foreign polypeptide chains such as B cell epitopes (10 to 15 amino acids) may be inserted into selected positions within two of the viral proteins, i.e.  
25 in exchange for one of the variable antigenic regions located at the surface of hemagglutinin (Muster et al., Mucosal model of Immunization against human immunodeficiency virus type 1 with a chimeric influenza virus. J. Virol. 69 (11), 6678-6686 (1995)), or into the stalk sequence of viral neuraminidase (Garcia-Sastre and Palese, The cytoplasmic tail of the  
30 neuraminidase protein of influenza A virus does not play an important role in the packaging of this protein into viral envelopes. Virus Res. 37, 37-47 (1995)), and be stably maintained as functional fusion proteins.

Constructs of this kind turned out to be useful for experimental vaccination in a few cases studied, but only rather few clearly defined epitope sequences (of ten to twelve amino acids each) are known today, and some of them might also be misfolded within such restricted fusion protein positions, or in other cases interfere with the correct tertiary structure and function of their host polypeptide chains.

Incorporation of a full-size foreign protein into influenza via reverse genetics, encoded by an independent ninth vRNA molecule in addition to its regular set of eight standard vRNA segments is without special provisions only transiently possible (Luytjes et al., Amplification, expression, and packaging of a foreign gene by influenza virus. Cell 59, 1107-1113 (1989); Enami et al., An influenza virus containing nine different RNA segments. Virology 185, 291-298 (1991)). In the absence of a continuous selective pressure any additional recombinant vRNA segment cannot be stably maintained as long as a wildtype promoter sequence is used on that ninth vRNA segment, and it will inadvertently be lost after few steps of propagation.

Using a different system of influenza reverse genetics developed in our laboratory (Zobel et al., RNA polymerase I catalysed transcription of insert viral cDNA. Nucleic Acids Res. 21, 3607-3614 (1993); Neumann et al., RNA polymerase I-mediated expression of influenza viral RNA molecules. Virology 202, 477-479 (1994)), which was built around *in vivo* synthesis of recombinant vRNA molecules by cellular RNA polymerase I transcription of the respective template cDNA constructs, promoter-up mutations have been designed by nucleotide substitutions (Neumann and Hobom, Mutational analysis of influenza virus promoter elements *in vivo*. J. Gen. Virol. 76, 1709-1717 (1995)). When these are attached to a recombinant ninth vRNA segment its increased transcription and amplification rate will not only compensate for the losses suffered spontaneously, but even cause accumulation of the foreign vRNA segment during simple viral passaging, in the absence of any selection. However, due to its over-replication relative to all of the regular influenza vRNA segments (which of

course are connected to wild-type promoter sequences) after catching up with the others the foreign segment will become over-abundant. This increasingly will result in viral particles that have incorporated several copies of recombinant vRNA, but no longer have a full set of all eight standard segments incorporated among an average of about 15 vRNA molecules present within a virion. Such particles are defective and will not result in plaque formation, hence after an initial increase of recombinant viral particles during the first steps of propagation a dramatic decrease is observed, usually at the third or fourth step of viral passaging, depending on the size of the recombinant vRNA and the level of the promoter-up mutation attached. A balanced situation with regard to the insert length and the level of promoter activity can be achieved, and has been propagated in a particular case over 11 passages, with essentially stable levels of recombinant viruses among a majority of helper viruses (around 80%) during these steps. If a full-level promoter-up mutation is used (1104 or the new variant 1920, see below) a balanced-level propagation is reached in conjunction with a recombinant vRNA size of 4000 nucleotides (Maysa Azzeh, Ph.D. Thesis, Univ. Giessen (2000)).

In all of these preparations, both in transiently achieved increased yields (up to 40% of recombinants after three or four steps of viral passage), and in a balanced propagation of recombinant influenza viruses (10 - 20%) the respective viral progeny inadvertently constitute mixtures with a majority of non-recombinant helper viruses. These result both from a statistical mode of packaging vRNA molecules into a virion (the ninth segment may not be co-packaged), and from the fraction of cells solely infected by helper virus.

The problems of fractional yields and of instability during viral propagation of recombinant influenza are the problems to be solved with the present invention.

## Summary of the Invention

Starting out from two observations in this laboratory which are discussed below and which concern two hitherto unsuspected properties of influenza viral RNA polymerase in its interaction with terminally adapted influenza-specific RNA molecules, a new technique for the construction of stable recombinant influenza viruses was found.

As previously described in WO 96/10641 plasmid constructs are designed to generate influenza vRNA-like molecules *in vivo* by cellular RNA polymerase I transcription following plasmid DNA transfection into tissue culture cells, and to this end contain flanking rDNA promoter and terminator elements, externally located relative to any cDNA constructs in between. The resulting recombinant vRNA molecules are designed to contain 5' and 3' recognition sequences for influenza viral RNA polymerase, which however carry up to five nucleotide substitutions (in promoter-up mutant pHL1920) resulting in a substantial increase of expression over wildtype influenza promoter levels. While recombinant pseudoviral RNA is initially transcribed by RNA polymerase I, further amplification and mRNA transcription depends on the presence of viral RNA polymerase and viral nucleoprotein in the cell, which generally are provided by infection of a helper virus. As a consequence the progeny viral yield will constitute a mixture of recombinant viruses together with a majority of wild-type helper viruses.

In the new technique the recombinant vRNA-like molecules as transcribed by RNA polymerase I are constructed as ambisense RNA double segments, with one reading frame (an influenza gene) in sense and a second one (a foreign gene) in anti-sense orientation, or vice versa. In such constructs both reading frames will be expressed via the cap-snatching mode, even if at different levels. Again, infection by helper virus is required to provide the necessary viral early and late proteins for genetic expression and virion packaging. However, the particular helper virus used in the new method is a recombinant virus carrying 2x2 specifically designed ribozyme targets inserted into the flanking non-coding sequences of one of its eight



vRNA segments. The viral segment chosen to become ribozyme-sensitive is always identical to the one used in constructing the recombinant ambisense RNA molecule, as the viral carrier gene in covalent linkage with an additional foreign gene.

- 5 Recombinant influenza viruses produced in this way through RNA polymerase I transcription of an ambisense viral RNA molecule followed by infection with that specifically designed type of ribozyme-sensitive helper virus will carry one of the influenza genes twice, once within that ribozyme-sensitive helper vRNA segment, and a second time within the
- 10 ribozyme-resistant ambisense segment. Recombinant viruses of this type are again obtained initially only as a mixture together with a majority of non-recombinant helper viruses. A progeny viral passage through tissue culture cells (293T) which before have been transiently transfected with plasmid constructs expressing the respective double-headed ribozyme will
- 15 (in one step) inactivate the ribozyme-sensitive segment by a factor of up to 100. One or two rounds of such ribozyme treatment *in vivo* will at the same time (a) purify the recombinant virus from its non-recombinant helper contaminants, and (b) delete the sensitive vRNA helper segment from within the initial (additive) recombinant virus.
- 20 As a result recombinant influenza viruses are isolated along this several-step procedure, which are free of contaminating helper viruses and carry seven regular and one ambisense vRNA segments, all in a balanced replication mode. Their recombinant nature is stably maintained because of a covalent junction between one of the viral genes and the full-size
- 25 foreign gene inserted, a situation achieved here for the first time; via constructing an influenza ambisense RNA segment. The whole procedure is independent of any (selectable) phenotype, and can be applied to either of the eight influenza vRNA segments. After establishing a first ambisense vRNA segment carrying a single foreign gene it can also be repeated all
- 30 over for inserting a second foreign gene within another ambisense RNA segment of the same constitution in principle.

Stable recombinant viruses of the type described can be used for cheap propagation in fertilized eggs, either for production of those recombinant viruses themselves or for production of foreign proteins or glycoproteins encoded by them, and hence find application in (glyco)protein production or in providing vector systems for somatic gene therapy or in being used as vaccination agents.

Thus, the present invention provides

(1) a recombinant influenza virus for high-yield expression of incorporated foreign gene(s), which is genetically stable in the absence of any helper virus and which comprises at least one viral RNA segment being an ambisense RNA molecule (hereinafter "ambisense RNA segment") and containing one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation, or *vice versa*, in overall convergent arrangement;

(2) a preferred embodiment of the recombinant influenza virus defined in (1) above, wherein one or more of the regular viral RNA segments, differing from said at least one ambisense RNA segment (hereinafter "modified regular segment"), comprises a vRNA encoding a foreign gene, preferably one or more of the regular viral RNA segments has (have) been exchanged for a vRNA encoding a foreign gene;

(3) a preferred embodiment of the recombinant influenza virus defined in (1) and (2) above, in which the terminal viral RNA sequences of said one or more modified regular segments and/or of said at least one ambisense RNA segment, which are active as the promoter signal, have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence;

(4) a method for the production of recombinant influenza viruses as defined in (1) to (3) above comprising

(a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, in antisense, sense or ambisense design,

- (b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in at least one of its viral RNA segments, and
- (c) thereafter selective vRNA inactivation through ribozyme cleavage;
- 5 (5) a method of constructing influenza carrier strains carrying one or more ribozyme target sites (of type one) in vRNA flanking positions comprising
- (a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, carrying two different 3' promoter sequences in tandem, which are separated by a second type of ribozyme target sequence, and which carry the said
- 10 internal ribozyme target sites of type one;
- (b) followed by infection of an influenza wildtype strain;
- (c) thereafter amplification through simple steps of viral propagation; and
- (d) finally isolation through removal of their external 3' promoter sequence by ribozyme cleavage through infection of cells expressing
- 15 ribozyme type 2, followed by plaque purification, said method being suitable for the construction of an influenza carrier strain required for step (b) of (4) above;
- (6) a ribozyme-sensitive influenza carrier (helper) strain obtainable by the method of (5) above;
- 20 (7) a pharmaceutical composition comprising a recombinant influenza virus as defined in (1) to (3) above;
- (8) the use of a recombinant influenza virus as defined in (1) to (3) above ~~for preparing a medicament for vaccination purposes;~~
- (9) the use of a recombinant influenza virus as defined in (1) to (3) above
- 25 for preparing agents for somatic gene therapy;
- (10) the use of a recombinant influenza virus as defined in (1) to (3) above for preparing agents for transfer and expression of foreign genes into cells (abortively) infected by such viruses;
- (11) the use of a recombinant influenza virus as defined in (1) to (3)
- 30 above for preparing agents for transfer and expression of RNA molecules into cells infected by such viruses;

(12) a method for the production of proteins or glycoproteins which comprises utilizing a recombinant influenza virus as defined in (1) to (4) above as expression vector;

5 (13) a method for preventing and/or treating influenza which comprises administering a recombinant influenza virus as defined in (1) to (3) above to the mammal to be treated, i.e., a vaccination method utilizing said recombinant virus;

10 (14) a method for somatic gene therapy, which method comprises subjecting the organism to be treated with a recombinant influenza virus as defined in (1) to (3) above;

(15) a method for transfer and expression of foreign genes into cells, and for transfer and expression of RNA molecules into cells, which method comprises infecting the cells with a recombinant influenza virus as defined in (1) to (3) above;

15 (16) use of a recombinant influenza virus as defined in (1) to (3) above for preparing agents for autologous immunotherapy;

(17) a method for an immunotherapy which comprises *ex vivo* infection of immune cells with a recombinant influenza virus as defined in (1) to (3) above, and introduction of the transduced cells into the patient; and

20 (18) a method for the induction of antibodies which comprises utilizing a recombinant influenza virus as defined in (1) to (3) above as an immunogen.

### **Brief Description of the Figures**

25 Fig.1 shows 3' nucleotide extensions of influenza vRNA template molecules.

Fig. 2 shows propagation of recombinant influenza viruses with tandem bicistronic vRNA.

30 Fig. 3 shows tandem bicistronic vRNA supporting an alternative mode of transcription and replication initiation

Fig. 4 shows the outgrowth of promoter-up recombinant vRNA versus wildtype vRNA segments in stepwise propagation of influenza virus.

Fig.5 shows pAM403 ribozyme cleavage of pHL2969 derived vRNA molecules at specific target sites inserted between an external and an internal 3' promoter sequence.

Fig. 6 shows comparative cleavage analysis of model CAT vRNAs with tandem target sites in various flanking positions, by target-specific ribozymes.

Fig. 7 shows an alignment of pAM424 double-headed ribozyme with one of their repetitive target sequences located within the 5' and 3' vRNA flanking regions.

10 Fig. 8 shows pAM424 ribozyme cleavage of resistant FPV wild-type HA vRNA and ribozyme-sensitive pHL2969-derived HA-vRNA in 293T cells.

Fig. 9 shows a functional analysis of the influenza cRNA promoter structure.

Fig.10 shows a functional analysis of the vRNA and cRNA promoter in ambisense arrangement.

Fig.11 shows a basepair substitution according to the vRNA 'corkscrew' structure.

Fig. 12 shows a flow-chart of the isolation procedure for an ambisense recombinant influenza virus.

20 Fig. 13 shows immuno-electron microscopy of purified influenza FPV/CSFV-E2-HA virions.

Fig. 14 shows vector pHL2969.

~~Fig. 15 shows vector pAM403.~~

Fig. 16 shows vector pAM424.

25 Fig. 17 shows vector pHL2507.

Fig. 18 shows vector pHL2583.

Fig. 19 shows vector pHL2989.

Fig. 20 shows vector pHL1920.

### 30 Detailed Description of the Invention

According to the present invention "influenza virus" embraces influenza A virus, influenza B virus and influenza C virus, with influenza A virus being

preferred. A "mammal" according to the present invention includes humans and animals. "Organism" embraces prokariotic and eukariotic systems as well as multicellular systems such as vertebrates (including mammals) and invertebrates. "Infected cells" and "infecting cells" according to the present invention also include "abortively infected cells" and "abortively infecting cells", respectively.

In a preferred influenza virus according to embodiment (1) at least one of the regular viral RNA segments is replaced by an ambisense RNA segment which contains one of the standard viral genes in sense orientation and a foreign, recombinant gene in anti-sense orientation or vice versa in overall convergent arrangement. It is moreover preferred that in the ambisense RNA molecule said foreign recombinant gene is covalently bound to one of the viral genes while the original vRNA segment coding for the same gene is deleted from the recombinant virus by a specific ribozyme cleavage.

The foreign gene(s) in ambisense covalent junction with the viral gene(s) preferably code for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus, such as lymphokines, or code for glycoproteins that are incorporated into the virion as well as the plasma membrane of the infected cell. In another preferred embodiment the foreign gene(s) in ambisense covalent junction with the viral gene(s) code for proteins or artificial polypeptides designed to support an efficient presentation of inherent epitopes at the surface of infected cells, for stimulation of B cell and/or T cell response. Such proteins or artificial polypeptides constitute for instance a tumor antigen or an artificial oligomeric series of T cell epitopes. Finally, the foreign gene(s) may be suitable for transfer and expression of RNA molecules, including antisense RNAs and double stranded RNAs, into cells. Such recombinant influenza viruses are suitable for sequence specific gene silencing, for example by antisense or RNA interference mechanisms.

A preferred recombinant virus of embodiment (2) of the invention is where in the regular viral RNA segments one or both of the standard glycoproteins hemagglutinin and neuraminidase have been exchanged, preferably into foreign glycoprotein(s) or into fusion glycoproteins consisting of an anchor segment derived from hemagglutinin and an ectodomain obtained from the foreign source, viral or cellular, or in which such recombinant glycoprotein has been inserted as a third molecular species in addition to the remaining standard components.

- 10 As set forth in embodiment (3) above, a preferred recombinant virus of the invention is where the terminal viral RNA sequences, which are active as promoter signal, have been modified by nucleotide substitution in up to 5 positions, resulting in improved transcription rates (of both the vRNA promoter and in the cRNA promoter as present in the complementary sequence) as well as enhanced replication and/or expression rates relative to the wild-type sequence. Said modified terminal viral RNA sequences differ from the wild-type sequence in that they are containing at least one segment (a naturally occurring segment or an additional segment) wherein the 12 nucleotide conserved influenza 3' terminal sequence has been modified by replacement of one to three nucleotides occurring in said sequence at positions 3, 5 and 8 relative to the 3' end by other nucleotides provided that the nucleotides introduced in positions 3 and 8 are forming a base pair (i.e., if the nucleotide position 3 is G, then that in position 8 is C; if the nucleotide in position 3 is C, then that in position 8 is G; etc.).

The 3' conserved regions of the wild-type influenza virus have the following sequences:

Influenza A: 5'-CCUGCUUUUGCU-3'

30 Influenza B: 5'-NN(C/U)GCUUCUGCU-3'

Influenza C: 5'-CCUGCUUCUGCU-3'.

Moreover, the 13 nucleotide conserved influenza 5'-terminal sequence may be modified by replacement of one or two nucleotides occurring in said sequence as positions 3 and 8 by other nucleotides, again provided that the introduced nucleotides are forming a base pair. The 5' conserved regions of the wild-type influenza virus have the following sequences:

Influenza A: 5'-AGUAGAAACAAGG

Influenza B: 5'-AGUAG(A/U)AACA(A/G)NN

Influenza C: 5'-AGCAGUAGCAAG(G/A):

- 10 Preferred influenza viruses of the invention are those wherein in the 3' conserved region the replacements G3A and C8U have been performed, more preferred are those where also the replacement U5C has been performed (the above mutations are relative to the 3' end; such counting from the 3' end is also indicated by a line on top of the digit, e.g., G  $\bar{3}$ A).
- 15 Another preferred influenza virus mutant comprises the 3'-terminal nucleotide sequence G3C, U5C and C8G (relative to the 3' end) giving the following 3' terminal nucleotide sequence 5'-CCUCGUUCUCCU-3'. Among the influenza viruses defined hereinbefore those having a 3'-terminal nucleotide sequence of 5'-CCUGUUUCUACU-3' are most preferred. In case
- 20 of an influenza A virus the segment may further have the modifications U3A and A8U in its 5' terminal sequence, in case of influenza C it may have the modifications C3U and G8A in its 5' terminal sequence. The most preferred influenza viruses of the present invention comprise the following general structures:

25 Influenza A (mutant pHL 1104):

5'-AGUAGAAACAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUC $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{C}}$ U $\overline{\text{A}}$ CU-3'

Influenza A (mutant pHL 1920):

5'-AG $\overline{\text{A}}$ AGAA $\overline{\text{U}}$ CAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUC $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{C}}$ U $\overline{\text{A}}$ CU-3'

Influenza A (mutant pHL 1948):

30 5'-AGUAGAAACAAGGNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'CCUC $\overline{\text{G}}$  $\overline{\text{U}}$  $\overline{\text{U}}$  $\overline{\text{C}}$ U $\overline{\text{C}}$ CU-3'



Influenza B:

5'-AGUAG(A/U)AACA(A/G)NNNNNU<sub>5-6</sub>..(880-2300 ntds)..N'N'N'N'(C/U)GUUUCUACU-3'

Influenza C:

5'-AGUAGUAACAAG(G/A)GU<sub>5-6</sub>..(880-2300 ntds)..CCCCUGUUUCUACU-3'

5

In the above structures the variables are defined as follows:

- (1) Underlined and enlarged letters show the required mutations relative to the wild-type sequence for preparing a promoter mutant with enhanced properties;
- 10 (2) enlarged A in the 5'-part of the sequence: additional A (position 10), angle-forming;
- (3) (A/G) at one position: different isolates or single segments with different sequence at this respective positions which are analytically interchangeable;
- 15 (4) N and N': undefined, but base paired positions relative to each other in complementarity between the 5' and 3' termini, different among the 8 segments, but constant for each segment throughout all viral isolates;
- (5) (880-2300 ntds): the lengths of the virus segments, in case of segments with foreign genes increased up to 4,000 nucleotides.

20

The pharmaceutical composition according to embodiment (7) above and the medicament of embodiment (8) above contains the recombinant influenza virus in a pharmaceutically effective amount. Besides said recombinant influenza virus, the pharmaceutical composition and the medicament may contain further pharmaceutically acceptable carrier substances well-known to a person skilled in the art, such as binders, desintegrants, diluents, buffers, preservatives, etc. The pharmaceutical composition and medicaments are solid or liquid preparations and are suitable to be administered orally, intravenously or subcutaneously.

30

The medicament according to embodiment (8) above is preferably suitable as a medicament against influenza and/or against other infections. The

recombinant influenza virus may be present in form of inactivated preparations or may be present in form of live recombinant viruses, preferably as attenuated viruses.

- 5 Live recombinant viral vaccines, live but attenuated recombinant viral vaccines or inactivated recombinant viral vaccine can be formulated. Inactivated vaccines are "dead" in the sense that their infectivity has been destroyed. Ideally, the infectivity is destroyed without affecting its immunogenicity. To prepare inactivated vaccines, the recombinant virus  
10 may be grown in cell cultures or in embryonated chicken eggs, purified, and inactivated by formaldehyde or  $\beta$ -propiolactone. The resulting vaccine is usually administered intramuscularly.

- Inactivated viruses may be formulated with a suitable adjuvant to  
15 enhance the immunological response. Such adjuvants may include, but are not limited to mineral gels, e.g., aluminum hydroxide, surface-active substances such as pluronic polyols, lysolecithin, peptides, oil emulsions, and potential useful human adjuvants such as BCG.

- 20 Many methods may be used to introduce the vaccine formulations above, for example the oral, intradermal, intramuscular, intraperitoneal, subcutaneous, or intranasal routes. Where a live recombinant virus vaccine is used, it may be preferable to introduce the formulation via the natural route of infection for influenza virus.

- 25 The medicament according to embodiment (8) above is preferably suitable for prophylactic or therapeutic vaccination, or both, against influenza and other infections. For example, recombinant viruses can be made for use in vaccines against HIV, hepatitis B virus, hepatitis C virus, herpes viruses, papilloma viruses, to name but a few. In one embodiment the  
30 recombinant virus contains the genes for surface proteins of the viruses, in another the genes for non-structural or regulatory genes. The

recombinant viruses may be present in form of inactivated preparations or may be present in form of live recombinant viruses, or in live, but attenuated viruses. In an attenuated virus the recombinant virus would go through a single or very few propagation cycle(s) and induce a sufficient level of immune response, but would not cause disease. Such viruses lack one of the essential influenza genes or contain mutations to introduce temperature sensitivity.

The agents of embodiments (9)-(11) of the invention are applicable in *ex vivo* and *in vivo* application schemes. The RNA molecule to be expressed by means of the agent of the embodiment (11) is of an antisense sequence or double strand sequence (in ambisense bidirectional transcription) relative to a target cellular mRNA molecule. In embodiment (11) the agent is preferably suitable for sequence-specific gene silencing, preferably by antisense RNA or RNA interference mechanisms.

The method for the production of proteins or glycoproteins is preferably performed in cell culture cells or in fertilized chicken cells in accordance with standard techniques within the general knowledge of a person skilled in the art. The proteins or glycoproteins to be expressed are those incorporated into the ambisense construct as defined hereinbefore.

The methods according to embodiments (13) to (15), (17) and (18) of the invention include the administration of an effective amount to the mammal or the administration of a sufficient infective dose of the recombinant virus to the cell system that is used for *ex vivo* therapy or for *in vitro* investigations, whereby the amount and dose will be determined by a person skilled in the respective arts or knowledgeable of the desired treatments.

The agent of embodiment (16) of the invention is preferably utilized to infect, transfect or transduce patient-derived immune cells. The agent is

suitable for treatment of cancer or chronic viral infections. For this purpose, patient derived immune cells, preferably dendritic cells, are *ex vivo* infected with recombinant influenza viruses expressing, e.g., tumor antigens or viral antigens. The transduced cells are then reintroduced into the patient.

The preferred method for immunotherapy of embodiment (17) of the invention is an autologous immunotherapy, wherein the cells which are *ex vivo* infected are patient-derived and the transduced cells are reintroduced into the patient. The diseases to be treated by this method include cancer and chronic viral infections. For details regarding such treatment see discussion of embodiment (16) above.

The method for inducing antibodies according to embodiment (18) of the invention is suitable for inducing antibodies to foreign proteins including glycoproteins, following the administration of protein or glycoprotein antigens as part of a recombinant influenza virus in an authentic conformation, whereby the virus is purified by gentle procedures based on hemagglutination, and the gene is expressed at high rates in the infected cells.

As influenza viruses have a wide host range, recombinant influenza viruses can be used to obtain strong immune responses in, and isolate antibodies from, a wide range of animals, including, but not limited to, fowl, pigs, goats, horses, and mice. Further, influenza viruses adapted to the mouse can be used for the infection of mice, by several routes including the intranasal route. This results in infection of the pharyngeal mucosal cells and results in an additional type of B cell response (e.g., as recognized in the ratio of IgG to IgA). Mice are of particular utility in the induction of immune responses in transgenic mice that have been engineered to express human antibodies. As gentle procedures based on hemagglutination are used to purify influenza viruses, antibodies to

antigens in native conformation can be isolated from the infected mammals.

Further preferred embodiments of the invention are set forth herein  
5 below.

A. Construction of influenza helper virus strains carrying ribozyme  
target sequences in flanking positions within either of the vRNA  
segments

10 A.1: Influenza RNA polymerase will initiate transcription and replication from promoter structures located at internal positions in an RNA molecule, not only from the natural position at both ends of a vRNA molecule:

This is true in particular for promoter-up variants in RNA-internal location  
15 due to their enhanced binding affinity for viral polymerase. Not only 3' end extensions are tolerated in RNA-internal promoter recognition (Fig. 1), but also 5' extensions as well as extensions at both ends of the RNA template molecule, containing noncomplementary as well as complementary sequence, i.e. potentially present as a double-stranded extension. Finally,  
20 also an extension by way of duplication of the promoter sequence (active or inactivated) leads to mRNA transcription and CAT expression, initiated from the active pair of 5' and 3' promoter halves, irrespective if in external or in internal or even in an oblique localization (active 5' promoter sequence in external, active 3' promoter sequence in internal position, or  
25 vice versa). RACE-determination of the resulting 5' and 3' ends of viral mRNA and cRNA, i.e. the products of transcription and replication reactions for several of the extended template vRNA constructs proves an exact recognition and sequence-specific initiation at a position equivalent to regular 3' position 1: all of the various template extensions are lost in  
30 every product RNA molecule, most likely after only one round of replication.

A.2: Bicistronic (tandem) vRNA molecules carrying an additional 3' specific promoter sequence in a central position between its two genes can be used for an indirect selection method for recombinant influenza viruses:

The method described is applicable for any foreign gene (e.g. CAT) without a selection potential of its own, if inserted into the distal mRNA position (proximal vRNA position, in anti-sense orientation) behind a carrier gene (e.g. GFP) in the proximal mRNA position, able to serve as a transient selection marker. The carrier gene which is used for selection will get lost spontaneously during further propagation. These constructs are equivalent to a 3' extension of the template vRNA by a full-size gene of 750 nucleotides up to a second 3' promoter sequence, in terminal location. While in the set of experiments shown in Fig. 2 the external 3' promoter sequence is maintained throughout as the same promoter-up variant (1104), the internal 3' promoter sequence has been varied to include a full-level promoter-up variant (pHL2270, containing promoter mutant 1104), a medium-level promoter variant (pHL2350, containing promoter mutant 1948), a wildtype promoter construct (pHL2629), and a construct carrying an unrelated central sequence in an otherwise identical design (pHL2300).

Due to the presence of two 3' promoter sequences in conjunction with a single 5' promoter sequence an alternating interaction between them will constitute one or the other active promoter structure (see Fig. 3). While the external location with an adjacent RNA 3' end may have a structural advantage, this appears to be compensated by the shorter distance in an interaction between the 5' sequence and the central 3' sequence in constituting the internal promoter, such that the competition between the two primarily reflects the various internal 3' promoter allele sequences used, compare Fig. 2B and activity ratios indicated above and below the lanes. Translation of the mRNA-distal gene (CAT) is only observed following an internal initiation at the bicistronic vRNA template, resulting in

a spontaneous deletion of the mRNA-proximal (vRNA-distal) gene, GFP, compare right half of Fig. 3. In complementary analyses GFP fluorescence is observed initially for all of the bicistronic constructs, but gets lost on a faster rate from pHL2270 transfected and influenza infected cells (not shown), and will stay unchanged in pHL2300-treated cells. The indirect selection system based on bicistronic (tandem) molecules as designed here and demonstrated for reporter genes GFP and CAT can be used for any other gene without distinct phenotype upon insertion behind an unrelated carrier gene with properties useful in selection. – In employing that technique an initial phase of (repeated) positive selection for infected cells expressing that proximal trait (e.g. by FACS or by magneto-beads) will be followed by a second phase with negative selection, i.e. against that fraction of infected cells still exposing the same property.

15 A.3: Isolation of an influenza strain designed to carry 2x2 flanking ribozyme target sequences at the 5' and 3' end of vRNA segment 4 coding for hemagglutinin:

The above scheme for an indirect selection of any foreign recombinant gene behind a proximal carrier gene is further modified by deleting the carrier gene altogether. Instead, both 3' terminal promoter sequences (mutant and wildtype) follow each other at a short distance, separated only by a specific, repetitive ribozyme target sequence, - different from other target sequences to be described further below. The cDNA insert following after the second 3' promoter sequence consists mainly of a regular hemagglutinin (H7) coding sequence, however both the 5' and 3' vRNA terminal regions of the insert carry that other ribozyme target sequence (different from the first target sequence mentioned above) inserted in either location in tandem duplication (pHL2969, see Fig. 14).

Due to a superior replication supported by the promoter-up variant located in 3' external position the recombinant HA segment attached to that promoter sequence will become enriched during the first steps of viral propagation, while the originally dominating HA segment of the helper

virus which is under control of a wildtype promoter sequence is consecutively reduced and finally is no longer detectable among viral progeny. This result is documented by RT-PCR analysis of consecutive viral populations as obtained in that stepwise propagation procedure, see Fig.

5 4.

In the next step the viral lysate is twice passaged via infection of cell culture cells (293T) that before have been DNA-transfected by plasmid pAM403 (Fig. 15). This construct has been specifically designed to express a hammerhead ribozyme with flanking sequences complementary to the  
10 repetitive GUC-containing target sequence, as present twice in between the external and internal 3' promoter signals in the recombinant HA vRNA segment, see Fig. 5. In this way the extra promoter sequence is cut off from the finally resulting recombinant HA vRNA. Its promoter-up activity was useful in achieving an initial increase in the concentration of  
15 recombinant HA vRNA over wildtype HA vRNA, and in finally excluding the latter from further propagation. However, for the same reason that high activity of the promoter variant will cause instability in the resulting viral progeny, and an effective 'substitution' at this time through ribozyme cleavage by the internally located promoter signal, wild-type or slightly  
20 improved, will restore stability to the progeny viruses, with all of their eight vRNA segments now brought back in balance to each other. Due to the ribozyme cleavage site at 26 nucleotides 3' of the wild-type promoter sequence (see Fig. 5), ~~in the initial stage that promoter signal is situated~~ in a vRNA-internal location, extended by a 3' terminal sequence of 26  
25 nucleotides. According to the data presented in Fig. 1 this should cause a transient slight reduction in activity, resulting however in one step in regular viral mRNAs and cRNAs, with any initially remaining extra sequence being lost from the finally resulting recombinant HA vRNA.

Progeny viruses still carrying an external promoter-up sequence (before  
30 ribozyme treatment or due to incomplete reaction) will not cause any plaque, due to over-replication of one vRNA segment relative to all others which results in a high load of defective particles. However, progeny



viruses which have lost that external promoter element due to ribozyme cleavage will yield regular plaques due to a balanced mode of replication for all eight wild-type or recombinant vRNA segments. Hence plaque purification is used for isolating a pure influenza viral strain carrying 2x2 ribozyme targets in its recombinant HA vRNA segment, with its termini reduced to the wild-type promoter sequence. The nature of the viral strain isolated has been confirmed in this regard by RT-PCR analysis, see Fig. 4.

The above isolation procedure resulting in influenza viral strains carrying 2x2 flanking ribozyme target sequences has been performed twice for the HA coding segment (segment 4) to obtain two different isolates with regard to the orientation of the ribozyme target sequences. In one of the isolates (vHM41, see SEQ. ID NO: 3) the tandem target sites have been inserted into the HA vRNA non-translated sequence both in 5' and 3' location, while according to the second design that 5' tandem target sequence has been included in an inverted orientation, such that it is now present in the cRNA 3' sequence instead (vHM42).

In another experiment the same procedure was used to isolate an influenza strain carrying 2x2 tandem target sites within the 5' and 3' flanking positions of segment 8 vRNA, i.e. coding for genes NS1 and NS2 (vHM81; see SEQ.ID NO:4). And in principle the same could be done for any other influenza segment, in particular since only the reading frame cDNA sequence has been exchanged from HA to NS, with all of the flanking elements directly responsible for that procedure remaining in place, unchanged.

A.4: Ribozyme cleavage and vRNA segment exchange using ribozyme-sensitive influenza strains as helper viruses:

In an initial model experiment a range of ribozyme type and target site locations was probed in designing a series of CAT reporter gene vRNA constructs (analysed in the presence of a surplus of wildtype helper virus) in 293T cells. While all of the ribozyme constructs adhered to the hammerhead model, with 10 to 12 nucleotides of complementary

sequence flanking on either side of the GUC target site, these ribozyme constructs varied from monomer to dimer to trimer repetitions. Ribozyme containing mRNAs were synthesized *in vivo* via the basic vector plasmid pSV2-*neo*, i.e. using the efficient p<sub>SVe</sub> RNA polymerase II promoter element for expression, and the SV40 origin signal for plasmid amplification, in a cell line (293T or cos-1) with an incorporated SV40 T antigen gene. In addition the pSV2-*neo* mRNA includes the small, 63 nucleotide intron sequence of the SV40 early mRNA which is supposed to be spliced very slowly, thereby extending the pre-mRNA half-life in the nucleus. Each of the pSV2-*neo*-ribozyme plasmid constructs was transfected into 293T cells. Thereafter, recombinant viruses containing dimer target sites either near one end of the molecule only, or near both ends have been used for infection of the transfected cells. Relative activities of ribozyme constructs versus vRNA target sites have been measured via inactivation of CAT acetyl transfer rates in the cell lysates obtained at 8 h post infection (Fig. 6). The highest activities were observed for dimer ribozymes acting on vRNA molecules carrying 2x2 target sequences on both ends of the molecule, either in vRNA 5' and 3' location, or in vRNA 3' and cRNA 3' location, i.e. with an inversion of the target site sequence at the vRNA 5' end.

Consequently, the two constructs carrying tandem ribozyme double targets within both of their non-translated vRNA flanking sequences have been used in the design of ribozyme-sensitive influenza virus strains as described above, with both variants isolated for segment 4 (HA), and one of them for segment 8 (NS). In complementary correspondence the hammerhead ribozyme plasmid used has also been constructed as a double-headed structure with flanking sequences as shown in Fig. 7 (pAM424; for its detailed structure, see Fig. 16).

The three target site-containing influenza strains isolated as described above have been analysed for their sensitivity against ribozyme cleavage by infection of 293T cells, which had been DNA-transfected 18 h earlier by

ribozyme-producing plasmid pAM424, at DNA-transfection rates between 60 and 70 % (as observed in parallel transfections using GFP-expressing plasmid pAM505). Inactivation rates in these one-step control experiments were between 90% and 99% for all three ribozyme-sensitive strains, in  
5 their extent mainly depending upon the actual DNA-transfection rates achieved in individual experiments.

In the next step both isolates of HA-coding ribozyme-sensitive viruses, vHM41 and vHM42, have been used in marker-rescue experiments. Here, 293T cells have been first DNA transfected by HA-variant cDNA construct  
10 pHL2507 (see Fig. 17), followed after 18 h by vHM41 or vHM42 virus infections at moi 1. The resulting viral supernatant containing e.g. a mixture of ribozyme-sensitive vHM41 carrier virus and pHL2507/vHM41 recombinant virus is propagated in an intermediate step on MDCK cells, which also results in an increase in the fraction of recombinant viruses.  
15 Thereafter the resulting virus-containing supernatant is passaged through 293T (or cos-1) cells, which in advance have been transiently transfected by ribozyme-producing pAM424. As may be concluded from the above experiment (Fig. 6) and shown in Fig. 8 vHM41- or vHM42-derived ribozyme-sensitive HA vRNA segments are expected to be inactivated by  
20 pAM424-produced ribozyme down to a remaining level of about 1% to 10% (mainly present within cells that are infected, but not DNA-transfected).

Instead, the substitute HA vRNA which originated from pHL2507 plasmid  
25 DNA (ribozyme-resistant) becomes exclusively incorporated into progeny virions. For further purification and viral propagation these have been passaged a second time through 293T cells, again in advance DNA-transfected by pAM424, and after another amplification step on MDCK cells the resulting viral preparations have been used for RT-PCR analysis.  
30 The resulting viral populations in these marker rescue experiments only contain HA-vRNA molecules derived from pHL2507, which in their PCR

analyses are of intermediate size relative to wildtype HA-vRNA, and vHM41- or vHM42-derived HA-vRNAs, respectively.

Consequently, a set of ribozyme-sensitive influenza strains with targets inserted individually into every vRNA molecule may be used for such one-step marker rescue experiments in general, i.e. for vRNA segment exchange reactions performed in a directional way for any of the eight influenza vRNA segments, without requirement for a selectable change in phenotype (genetic marker).

10 B. Expression of two gene products from ambisense bicistronic influenza vRNA

B.1: The influenza cRNA promoter is active in antisense viral mRNA transcription according to the cap-snatching mode of initiation:

15 While the vRNA template of influenza virus is known to be active in viral mRNA as well as cRNA synthesis, the cRNA template has been described so far only to produce vRNA molecules, as a second step in viral replication. The potential activity of the cRNA promoter in initiating also viral mRNA transcription has not been analysed or even suspected so far, 20 since no antisense (vRNA) reading frame can be detected in any of the viral RNA segments. Also, no U<sub>5</sub>/U<sub>6</sub> template sequence element is present in any of the viral cRNAs in an adjacent position to its 5' promoter structure as is the case for all viral vRNAs. This element is known to serve as a template sequence for mRNA terminal poly-adenylation, in repetitive 25 interaction. However, when both elements are provided through reconstruction of an artificial influenza cRNA segment: a reading frame in opposite orientation (CAT), and a U<sub>6</sub> template element in 5' adjacent location, CAT expression can indeed be observed, see pHL2583 (see Fig. 18) in Fig. 9. Similar to the vRNA promoter the cRNA promoter activity is 30 improved by (the same) promoter-up mutations, which essentially consist of basepair exchanges according to the 'corkscrew' model. This model apparently also holds for the cRNA promoter structure as analysed in a

stepwise manner in Fig. 9. While the cRNA promoter has to be superior over the vRNA promoter in its initiation of replication, since the vRNA/cRNA product ratio was determined to be around 10:1 (Yamanaka et al., *In vivo* analysis of the promoter structure of the influenza virus RNA genome using a transfection system with an engineered RNA. Proc. Natl. Acad. Sci. USA 88, 5369-5373 (1991)), the cRNA promoter is observed to be inferior to the vRNA promoter in its initiation rate of transcription (compare pHL2583 with pHL1844 in Fig. 9), at least for all promoter variants tested so far.

A RACE analysis for determination of the 5' ends in pHL2583 cRNA promoter transcribed mRNAs proved this initiation to occur according to the cap-snatching mode, in complete equivalence to standard vRNA promoter controlled transcription initiation.

B.2: Development of ambisense influenza constructs for consecutive expression of two genes (GFP and CAT) from a single viral RNA:

For a bidirectional transcription and translation of influenza RNA segments the two reporter genes GFP and CAT have been arranged in opposite orientation to each other, and the flanking 5' and 3' promoter sequences

(adhering to promoter-up variant 1104) had to be reconstructed to include a U<sub>6</sub> poly-adenylation element in either orientation in a 5' promoter adjacent position. This requirement necessarily resulted in a promoter-adjacent 5'-U<sub>6</sub>/3'-A<sub>6</sub> complementary structure, both in the vRNA and cRNA terminal sequence (see Fig. 10), which had to be tested for its promoter

activity, in either orientation. Therefore, the convergent pair of reporter genes GFP and CAT has been inserted in both orientations, such that CAT transcription is initiated by the vRNA promoter in one construct (pHL2960), and by the cRNA promoter in the other (pHL2989, Fig. 19), and vice versa for GFP expression from both ambisense constructs. In

addition, also the CAT gene only has been inserted in either orientation between the 5' and 3' elements of that ambisense promoter, with CAT transcribed by the vRNA promoter in one case (pHL2959), and by the

cRNA promoter in the other (pHL2957). The whole set of constructs allows for a direct comparison with corresponding reference constructs carrying a regular vRNA promoter (pHL1844) or cRNA promoter structure (pHL2583), i.e. carrying only the 5'-adjacent U<sub>6</sub> sequence element and no 3'-A<sub>6</sub> counterpart. The two groups of constructs also differ in insert size, since a single inserted gene roughly accounts for 750 nucleotides, the convergent set of two genes for 1500 nucleotides, with the distal half of both mRNAs in this case remaining untranslated, a situation unusual for influenza viral mRNAs.

As is demonstrated in Fig. 10B for CAT expression of the various ambisense constructs all of them are able to initiate transcription in both orientations, even if at different levels with regard to their vRNA and cRNA promoter-dependent expressions, and also with regard to the insert lengths and convergent arrangements of the GFP/CAT versus CAT-only constructs. Analysis of the GFP expression rates (not shown) yields complementary results, i.e. again vRNA promoter-controlled GFP expression is superior over cRNA promoter expression of GFP. Therefore, individual ambisense clones either show an asymmetric high expression of GFP and low expression of CAT (pHL2960) or vice versa (pHL2989), depending on their orientation of reading frames with regard to the external vRNA and cRNA promoter. Fig. 10C also demonstrates successful propagation of recombinant viruses containing ambisense RNA molecules, which proves survival through amplification, packaging into virions, and expression of both mRNAs in infected MDCK cells (including besides CAT also GFP expression).

B.3: Construction of a superior promoter-up mutation, pHL1920, to be used for improved rates of cRNA promoter expression in ambisense constructs:

An extended analysis of promoter variants, in particular of complementary double exchanges according to the 'corkscrew' model yielded among others variant pHL1920 (Fig. 20) with CAT activity rates considerably

above (125-130% of) the rates observed for standard promoter-up variant '1104' (as present in pHL1844). The '1920' promoter-up variant consists of altogether 5 nucleotide substitutions relative to the wildtype promoter sequence, both in the 5' promoter element (2), and the 3' promoter element (3). The structure of this variant and the whole set of complementary double exchanges is presented in Fig. 11, together with the respective CAT activity measurements, in vRNA promoter constructs. vRNA promoter-up variants also show similarly improved expression in (ambisense) cRNA constructs, even if at generally lower levels than in vRNA constructs. cRNA promoter-up expression is observed at levels similar or somewhat (2x-5x) above the *wild-type* vRNA promoter rate, while vRNA promoter-up constructs show CAT expression rates increased up to 20 or 25 times the wild-type vRNA promoter level. In either case expression rates also depend on the size of the insert, with promoter activity rates decreasing with increasing lengths of the influenza RNA molecules to be transcribed.

B.4: Influenza recombinant viruses containing a foreign gene (CAT) in covalent ambisense linkage with one of the viral genes (HA, NS1/NS2):

The principle solution in designing stable recombinant viruses based on the new properties observed for influenza transcription and replication signals consists in constructing viruses which contain a foreign gene in covalent linkage with one of the (indispensable) viral genes, in ambisense bicistronic organization. Preferably the viral gene is connected to the cRNA promoter, while vRNA promoter expression is used for expression of the foreign gene at rates considerably above the viral mRNA synthesis. The promoter-up variant chosen for constructing the ambisense RNA segment intends to bring its cRNA promoter expression (approximately) into balance with all other viral gene expression levels, which are controlled by wild-type vRNA promoters located at the termini of the seven ordinary influenza segments; the respective choice has to take into consideration the overall length of the ambisense segment.

Isolation of the ambisense recombinant virus employs an RNA polymerase I-transcribed ambisense cDNA construct, which will give rise *in vivo* to ambisense cRNA-type molecules, see Fig. 12. The plasmid DNA transfection mixture used in this step with 293T cells in addition may or may not contain four 'booster' plasmids which under p<sub>CMV</sub>-control produce the four early influenza proteins from non-viral mRNAs: NP, plus PB1, PB2, and PA, i.e. the three subunits of viral polymerase (Pleschka et al., A plasmid-based reverse genetics system for influenza A virus J. Virol. 70, 4188-4192 (1996)), which will increase in a pre-amplification step the copy number of that ambisense viral cRNA segment. At 18 h post transfection the 293T cells are infected by a ribozyme-sensitive influenza strain, e.g. vHM41, which will supply (again) early and also late viral RNAs. The resulting supernatant which contains a mixture of vHM41 carrier virus and vHM41-derived ambisense recombinant virus (nine vRNA segments) is then passaged directly or via an intermediate step of amplification on MDCK cells onto 293T cells that have in advance been DNA-transfected by ribozyme-producing pAM424. Here, the ribozyme-sensitive vRNA segment of vHM41 will be cleaved at its 2x2 target sites by pAM424 specific ribozymes. In recombinant viruses the vRNA gene lost in this way is re-supplemented through its presence within the ambisense segment. The virus-containing supernatant is passaged for amplification and further purification through ribozyme treatment a second time on 293T cells which again have been pretreated by pAM424 DNA transfection. Absence of ribozyme-sensitive vRNA, and presence only of ambisense RNA in RT-PCR analysis at this stage allows for further amplification on MDCK cells and a final virus stock preparation on embryonated chicken eggs. CAT assays can be used to analyse for the presence and monitor the activity of this model foreign gene through the various steps of isolation and propagation as well as document technical improvements that might be worked out for one or more of the processive stages.



C. Examples for application of helper-free, stable recombinant influenza viruses

C.1: Incorporation of reporter gene GFP in NS/GFP or HA/GFP ambisense segments:

Recombinant viruses of this type will allow to follow-up on influenza infection instantly and continuously in individual infected cells, which may also be counted or documented by fluorescence photography or FACS sorting. With improved temperature resistance (Siemering et al.,  
Mutations that suppress the thermosensitivity of green fluorescent protein. Curr. Biol. 6, 1653-1663 (1993)) GFP expression becomes visible at 2 h after infection and shows bright fluorescence after 4 h p.i., it will be possible to follow-up on the spread of viral infection by GFP fluorescence. Stable fluorescence in abortively infected cells e. g. observed in ex vivo treatment of dendritic cells similarly supports a follow-up on their reincorporation into animals; other genes may be incorporated by ambisense vRNA into dendritic cells in the same way.

C.2: Construction of glycoprotein CSFV-E2 carrying influenza, helper-free:

The glycoprotein E2 of CSF virus has been incorporated previously into influenza both as an HA-anchor fusion protein within the viral envelope, and as an additional, unstable ninth vRNA segment into its genome (Zhou et al.; Membrane-anchored incorporation of a foreign protein in recombinant influenza virions. Virology, 246, 83-94 (1998)). Stabilization is now achieved through an ambisense connection with either of the regular viral RNA segments (NS or HA) which also allowed to reach a level of 100% recombinant viruses instead of an hitherto only 20% (Fig. 13), since all carrier viruses are destroyed through ribozyme action, see Figs. 8 and 12. The helper virus containing preparation has already been used successfully as a vaccine against CSFV infection (antibody titers of 1:40000); the increase achieved in recombinant viruses allows a further

improvement in that regard. Also (cost-effective) propagation in fertilized chicken eggs has become possible due to its stable incorporation of the foreign gene as a covalent ambisense construct.

5 C.3: Construction of hepatitis C glycoprotein-carrying recombinant influenza viruses as a candidate vaccine:

Hepatitis C virus is a close relative of CSF virus (hog cholera virus), and in particular its set of two glycoproteins, small-size E1 and larger-size E2, is closely related in structural detail and presumably also in function to the  
10 corresponding CSFV proteins. An incorporation of HCV-E2/HA fusion proteins into influenza viral envelopes has been achieved in analogy to the CSFV-E2/HA incorporation. In addition, incorporation of an anchor-fusion glycoprotein HCV-E1/HA or both together (in NS *and* HA ambisense junctions) allows further variations in constructing an influenza-based  
15 vaccine for hepatitis C. In analogy to CSFV-E2, neutralizing antibodies are expected to be directed against particular epitopes of HCV-E2, presented in essentially native conformation at the influenza viral envelope.

20 C.4: Stable incorporation of selected influenza T-cell epitopes in ambisense constructions:

Influenza infection is known to result in both, antibody production against that specific viral strain or indeed its epitopes that are located mainly at  
~~the surface of HA, and in an increase of specifically primed cytotoxic T-~~  
lymphocytes, stimulated by T-cell epitopes primarily located within the  
25 essentially invariable core structure of the NP protein. While the humoral response will result in life-long immunity against *that particular* strain of influenza or its epitope structures, the T-cell response will be lost or severely reduced some time afterwards, such that its specificity against influenza in general will fall below protective levels. One way in trying to  
30 increase that level of cellular immunity is to enhance the response or recruitment of influenza-specific CTL cells by increasing the level of T-cell epitopes in the infected cell and hence its presentation on the surface by

MHC-I receptors. This is achieved by combining in an ambisense construct the HA gene and a series of repeated T-cell epitope sequences as present in the influenza NP gene, in a model design specific for the BALB/C mouse MHC-I allele. Here, the promoter-up expression rate is realized (in vRNA promoter-controlled initiation) for expression of the repetitive epitope polypeptide chain. Alternatively or in addition a controlled secretion of an interleukin can be achieved from recombinant influenza-infected cells, upon ambisense incorporation of the respective gene preferably into the NS segment. The interleukin to be chosen for this purpose (IL-12 or other) is selected to enhance the longevity of influenza-specific CTL cells or its conversion into corresponding memory cells. In this way an ambisense vaccine strain against influenza itself is achieved with expected protective capacity against influenza in general.

15 C.5: Exchange of influenza glycoproteins against foreign viral glycoproteins (VSV-G):

The 'marker rescue' experiment described above (section A.4.), i.e. an exchange of one HA gene (ribozyme-sensitive) for another (ribozyme-resistant) can also be used for an exchange of HA for an entirely different glycoprotein, such as the vesicular stomatitis virus G protein, as long as it is attached to the HA anchor segment. Effective incorporation depends always on that C-terminal tail sequence and its interaction with underlying matrix protein M1, and therefore, all constructions consist of fusion proteins in direct analogy to CSFV-E2. VSV-G with or without a foreign anchor sequence has been shown in several other viruses to be able to substitute for the original glycoprotein and to result in infectious viruses with VSV-G specific host-ranges (e.g. in retroviruses, rabies virus, measles virus). G protein in VSV itself as well as on the surface of foreign viruses is the only glycoprotein required for all of the consecutive steps in infection.

30 Insertion of VSV-G instead of HA in recombinant influenza viruses leaves the second glycoprotein, neuraminidase, without any function, which then will get lost spontaneously from the recombinant viruses. This will further

increase the capacity for an addition of foreign genes, beyond the gain resulting from an exchange of the larger HA for the smaller VSV-G, which might be used for an addition of ambisense constructs.

- 5 The invention is further illustrated in the accompanying figures.

### Detailed Description of the Figures

Fig.1: 3' nucleotide extensions of influenza vRNA template molecules:

- 10 (A) Murine B82 cells have been transfected by plasmid cDNA constructs designed to be transcribed into influenza vRNA molecules by RNA polymerase I *in vivo*, followed after 20 h by standard FPV<sub>Bratislava</sub> helper virus infection, at an moi of 1 to 3. In addition to reference plasmid pHL2024 (no extension), related cDNA constructs carrying extensions of 1  
15 to 50 bp, and hence extended by 1 to 50 nucleotides at the resulting vRNA 3' ends were used in parallel transfections; template extensions are marked at the top of the figure. Cell lysates prepared at 8 h post helper virus infections were used for CAT reactions using in one round 50 µl of cell lysate each, and in further analyses 5µl and 0.5 µl of lysate (not  
20 shown). Relative yields were determined in comparison to reference plasmid pHL2024, as indicated below the figure, with calculations restricted to those CAT assays showing less than 40% of substrate consumption, in three or more independent experiments.

- (B) Viral passage of B82 supernatants containing recombinant influenza  
25 virus onto MDCK cells, at an moi of 2 to 4, in average. Again at 8 h post infection cell lysates have been prepared and used for CAT assays. Relative yields as indicated below the figure have been determined in comparison to pHL2024 the same way as in (A), using 0.5 µl of cell lysate in each case. (The 50 µl CAT assays as shown here and also in the  
30 following figures intend to give an immediate impression of relative activities at always the same level, while the actual measurement data as

indicated below the lanes are obtained at various appropriate enzyme concentrations relative to reference pHL2024.)

Fig. 2: Propagation of recombinant influenza viruses with tandem bicistronic vRNA:

(A) General design of expression plasmids for transient bicistronic vRNAs coding for GFP in the mRNA-proximal, and for CAT in the mRNA-distal position. Among the functional elements indicated are the human RNA polymerase I promoter ( $p_{IH}$ ) and murine rDNA terminator ( $t_I$ ) sequences, both hatched, and the 5' and 3' vRNA promoter cDNA sequences, open and closed boxes, respectively. For the vRNA-internal 3' promoter signal three variant sequences have been inserted as indicated below (positions 1 to 15 refer to 3'-terminal nucleotides in the resulting monocistronic vRNAs).

(B) CAT assays as determined relative to pHL1844 (monocistronic CAT construct) after DNA transfection of 293T cells plus helper virus infection followed by one round of progeny viral propagation on MDCK cells are indicated *below* the lanes. Relative activities of the internal promoter sequences as indicated *above* the figure refer to measurements in a monocistronic *external* location of the same promoter variants (Flick *et al.*, Promoter elements in the influenza vRNA terminal structure, RNA 2, 1046-1057 (1996)). Control clone pHL2300 contains an unrelated, non-functional sequence in the central location in an otherwise identical plasmid construct.

Fig. 3: Tandem bicistronic vRNA supporting an alternative mode of transcription and replication initiation:

An additional internal 3' promoter sequence has been inserted in between both cistrons, in a vRNA-central position. Left half: bicistronic replication and transcription leading to (proximal) GFP expression. Right half: internal initiation resulting in monocistronic replication and transcription leading to

(distal) CAT expression, and causing deletion of the GFP sequence from progeny molecules.

Fig. 4: Outgrowth of promoter-up recombinant vRNA versus wildtype vRNA segments in stepwise propagation of influenza virus:

RNA polymerase I transcription of transfected pHL2969 DNA results in influenza vRNA carrying (an external) promoter-up mutant '1104', and containing 2x2 ribozyme targets in flanking positions relative to its HA coding sequence. Another HA vRNA segment in wild-type configuration and originating from infecting FPV helper virus is also present in the recombinant virus preparation, initially (lane 1; 293T lysate) in surplus amounts, but reduced and finally lost entirely in consecutive steps of propagation (lanes 2 to 4; MDCK cell lysates), and in isolated strains after pAM403 ribozyme treatment for removal of the external '1104' promoter sequence (lane 5). Determination throughout by RT-PCR analyses using a pair of primers extending across the 5' inserted target site sequence, with 435 bp representing the recombinant HA segment, and 306 bp the wild-type sequence without an inserted target site sequence.

Fig.5: pAM403 ribozyme cleavage of pHL2969 derived vRNA molecules at specific target sites inserted between an external and an internal 3' promoter sequence:

The external promoter-up ('1104') signal is used for vRNA amplification within recombinant viruses and reduction of helper virus HA vRNA (Fig. 4), while the 'switch' to an internal wild-type signal guarantees stable replication of recombinant viruses. pAM403 hammerhead ribozyme RNAs are indicated in complementary binding to their target site sequences (12 and 10 nucleotides flanking the GU'C cleavage point) by straight lines flanking a central secondary structure symbol. vRNA-internal 2x2 ribozyme targets are marked by xx (see Fig. 7).

Fig. 6: Comparative cleavage analysis of model CAT vRNAs with tandem target sites in various flanking positions, by target-specific ribozymes:

293T tissue culture cells have been transiently DNA-transfected either by a single-headed hammerhead ribozyme (**s**), or a double-headed (**d**), or triple-headed (**t**) ribozyme cDNA construct, all specifically designed to hybridize to a tandem dimer target site sequence inserted in flanking positions into the CAT vRNA. All ribozyme RNAs have been expressed from the same pSV2-*neo* plasmid vector, including a pSV2-*neo* control construct without an inserted ribozyme cDNA sequence (**c**). At 20 h after DNA transfection (which reached 65% yield as measured by p<sub>CMV</sub>-GFP transfection in parallel of the same cell culture) the 293T cells were infected by CAT recombinant viruses carrying tandem double target sequences either only in vRNA-3' position, or in both vRNA-3' and 5' positions, or in both vRNA-3' and cRNA-3' positions. Most effective among the s, d, or t-ribozymes were double-headed constructs, acting on 2x2 targets inserted in either of the two localizations described (lanes 6 and 10).

Fig. 7: Alignment of pAM424 double-headed ribozyme with one of their repetitive target sequences located within the 5' and 3' vRNA flanking regions:

The superior activity of ribozymes oriented against targets located in the 3' end of vRNA molecules over those present in the 5' end instead (not shown) is in agreement with the model for influenza vRNA transcription and replication (Lamb and Krug, Orthomyxoviridae: The viruses and their replication. In 'Virology' (B.N.Fields, D.M.Knipe, P.M.Howley, R.M.Chanock, J.L.Melnick, T.P.Monath, B.Roizman, and S.E.Straus, Eds.), 3<sup>rd</sup> ed., Vol. 1, pp. 1353-1395. Lippincott-Raven, Philadelphia (1996)), according to which influenza polymerase stays attached to the 5' end of the vRNA molecule throughout the entire or even several rounds of transcription, whereas the very 3' end repeatedly, in every initiation reaction serves as the template sequence, and consequently is no longer covered by polymerase.

Superiority of a double-headed over a single-headed ribozyme has been determined earlier in this laboratory (A.Menke, Anti-Influenza Ribozyme: vRNA-Spaltung und intrazelluläre Aktivität. Dissertation Universität Giessen (1997)), but the substantial increase of vRNA inactivation rates upon incorporation of tandem target sites at both ends of the vRNA molecule instead of only one has been observed here for the first time, within that overall design.

Fig. 8: pAM424 ribozyme cleavage of resistant FPV wild-type HA vRNA and ribozyme-sensitive PHL2969-derived HA-vRNA in 293T cells infected by vHM41 after isolation from PHL2969-recombinant viral preparations. Lane 1: FPV infection of 293T cells, untreated; lane 2: FPV infection of 293T cells DNA-transfected by pAM424; lane 3: vHM41 infection of 293T cells, untreated; lane 4: vHM41 infection of 293T cells DNA-transfected by pAM424. RT-PCR analyses of purified viral progeny as in Fig. 4.

Fig. 9: Functional analysis of the influenza cRNA promoter structure:

(A) Schematic cRNA promoter ('1104') secondary structure according to the 'corkscrew' model; nucleotides involved in single or double nucleotide exchange are marked by their position.

(B) CAT analyses of 293T cell lysates after DNA transfection and FPV helper virus infection of cRNA promoter variants, in comparison to standard vRNA promoter-up mutant '1104' (pHL1844). Nucleotide substitutions divergent from the basic '1104' structure as present in PHL2583 or PHL2721 (see above) are indicated above the lanes, positions 3 or 8 as marked by a bar refer to cRNA positions counted from the 3' end. Relative CAT activities are marked below the lanes.

Fig.10: Functional analysis of the vRNA and cRNA promoter in ambisense arrangement:



(A) Sequence organisation of the ambisense promoter cDNA construct carrying T<sub>6</sub>/A<sub>6</sub> elements adjacent to the terminal sequence, and secondary structure predictions for the resulting cRNA and vRNA promoter signal.

(B) CAT expression data obtained from the cell lysates of 293T cell after plasmid DNA transfection and FPV infection, and (C) from cell lysates of MDCK cells after one step of viral passage. Indicated above the lanes are promoter/gene conjunctions: v = vRNA promoter; c = cRNA promoter.

Fig.11: Basepair substitutions according to the vRNA 'corkscrew' structure:

(A) 'Corkscrew' conformation of the vRNA promoter drawn against a schematic indication of interacting tripartite viral polymerase. Paired positions exchanged in individual experiments are indicated by numbers, nucleotides  $\bar{3}$  or  $\bar{8}$  are counted from the 3' end. pHL2024 containing promoter-up mutation '1104' is used as the reference construct (=100%) in all of the CAT assays, while pHL2428 represents the wild-type promoter structure.

(B) CAT expression data obtained after one step of viral passage in MDCK undiluted, and 50 fold diluted.

Fig. 12: Flow-chart of the isolation procedure for an ambisense recombinant influenza virus.

Fig. 13: Immuno-electron microscopy of purified influenza FPV/CSFV-E2-HA virions:

Recombinant viruses exposing the foreign glycoprotein CSFV-E2 in their envelopes, which has been fused onto the HA anchor domain, are marked by anti-E2 monospecific antibody and by secondary gold-labelled (5nm) goat antibody. Recombinant viruses (16%) are present together with their FPV helper viruses.

Fig. 14: pHL2969; the exact sequence of the 4930 bps circular DNA is shown in SEQ. ID NO:1.

Fig. 15: pAM403; the exact sequence of the 5811 bps circular DNA is shown in SEQ. ID NO:2.

- 5 Fig. 16: pAM424; the exact sequence of the 5860 bps circular DNA is shown in SEQ. ID NO:5.

Fig. 17: pHL2507; the exact sequence of the 4610 bps circular DNA is shown in SEQ. ID NO:6.

10

Fig. 18: pHL2583; the exact sequence of the 3558 bps circular DNA is shown in SEQ. ID NO:7.

- 15 Fig. 19: pHL2989; the exact sequence of the 4343 bps circular DNA is shown in SEQ. ID NO:8.

Fig. 20: pHL1920; the exact sequence of the 3888 bps circular DNA is shown in SEQ. ID NO:9.

- 20 Hence, the present invention is based on two surprising findings, namely  
1. influenza virus promoters are active when present internally in a gene;  
2. the so-called cRNA, thought to be an intermediate in replication can be turned into a protein-encoding RNA by equipping it with a variant influenza virus promoter, described in the present invention.

25

- These two observations were used to make ambisense constructs. This allows to package an additional, foreign gene into influenza virus particles. Such particles were made previously, by other methods, but proved to be unstable, and therefore useless. For use as a vaccine for example, a  
30 helper virus would have been needed as a stabilizer. Stabilization in the present invention is achieved by several means. These include the "balancing" of one of the two promoters in the ambisense biscistronic

genetic construct with seven other vRNA wildtype promoters, while the additional promoter is used for high-rate expression of the foreign gene at various levels.

- 5 Thus, the present invention provides a system for expression of foreign proteins in higher eukaryotic systems. One system in particular is interesting, namely embryonated chicken eggs, as it allows cost-effective production in an automatable way (as used by most flu vaccine producers). The reason that this process is now possible, is that the  
10 foreign protein is part of a stable, engineered influenza virus particle. The virus can be designed also to rapidly monitor process improvements.

- An excellent use is of course the use of the construct as a vaccine. The influenza virus particle is immunogenic and can now be equipped with  
15 foreign antigens, enabling for example the design and production of hepatitis C virus and HIV vaccines, but also of tumor vaccines. As the present invention shows, the foreign antigenic surface glycoprotein is "fused" to a C-terminal segment of influenza HA, and the antigen then is presented at the surface of influenza virus particles. In addition, these  
20 vaccines can now be made in the way standard flu vaccines are made, i.e., in embryonated chicken eggs.

## Claims

1. A recombinant influenza virus for high-yield expression of incorporated  
5 foreign gene(s), which is genetically stable in the absence of any helper  
virus and which comprises at least one viral RNA segment being an  
ambisense RNA molecule (ambisense RNA segment) and containing one of  
the standard viral genes in sense orientation and a foreign, recombinant  
gene in anti-sense orientation, or *vice versa*, in overall convergent  
10 arrangement.
2. The recombinant influenza virus of claim 1, wherein at least one of the  
regular viral RNA segments is replaced by an ambisense RNA segment  
which contains one of the standard viral genes in sense orientation and a  
15 foreign, recombinant gene in anti-sense orientation, or *vice versa*, in  
overall convergent arrangement.
3. The recombinant virus according to claims 1 and 2, wherein in the  
ambisense RNA molecule said foreign recombinant gene is covalently  
20 bound to one of the viral genes, while the original vRNA segment coding  
for the same gene is deleted from the recombinant virus by way of specific  
ribozyme cleavage.
4. The recombinant influenza virus according to claims 1 to 3, wherein  
25 one or more of the regular viral RNA segments, differing from said at least  
one ambisense RNA segment, comprises a vRNA encoding a foreign gene,  
preferably one or more of the regular viral RNA segments has (have) been  
exchanged for a vRNA encoding a foreign gene.
- 30 5. The recombinant influenza virus according to claim 4, in which one or  
both of the standard glycoproteins hemagglutinin and neuraminidase have  
been exchanged into foreign glycoprotein(s) or into fusion glycoproteins

consisting of an anchor segment derived from hemagglutinin and an ectodomain obtained from the foreign source, viral or cellular, or in which such recombinant glycoprotein has been inserted as a third molecular species in addition to the remaining standard components.

5

6. The recombinant influenza virus according to claims 1 to 5, in which the terminal viral RNA sequences of one or more of the regular segments and/or of the at least one ambisense RNA segment, which are active as the promoter signal, have been modified by nucleotide substitutions in up to five positions, resulting in improved transcription rates of both the vRNA promoter as well as the cRNA promoter as present in the complementary sequence.

7. The recombinant influenza virus of claim 6, wherein the 12 nucleotide conserved influenza 3' terminal sequence has been modified by replacement of one to three nucleotides occurring in said sequence at positions 3, 5 and 8 relative to the 3' end by other nucleotides, and/or wherein the 13 nucleotide conserved influenza 5' terminal sequence has been modified by replacement of one or two nucleotides occurring in said sequence at positions 3 and 8 by other nucleotides.

8. The recombinant influenza virus of claim 7, wherein the replacements in the 3' terminal nucleotide sequence comprises the modifications G3A and C8U.

25

9. The recombinant influenza virus of claim 8, wherein the replacements in the 3' terminal nucleotide sequence comprises the modifications G3A, U5C and C8U, or G3C, U5C and C8G.

10. The recombinant influenza virus of claim 9, which comprises a 3' terminal nucleotide sequence of 5'-CCUGUUUCUACU-3'.

11. The recombinant influenza virus of claims 7 to 10, wherein the 5' terminal nucleotide sequence comprises the modifications U3A and A8U resulting in a 5'-terminal sequence of 5'-AGAAGAAUCAAGG.

5 12. The recombinant influenza virus according to claims 1 to 11, which is a recombinant influenza A virus.

13. The recombinant influenza virus according to claims 1 to 12, in which the foreign gene(s) in ambisense covalent junction with viral gene(s) code  
10 for proteins and/or glycoproteins which are secreted from cells infected with the recombinant virus.

14. The recombinant virus according to claims 1 to 12, in which the foreign gene(s) in ambisense covalent junction with viral gene(s) code for proteins  
15 or artificial polypeptides designed to support an efficient presentation of inherent epitopes at the surface of infected cells, for stimulation of a B cell and/or T cell response.

15. A method for the production of recombinant influenza viruses as  
20 defined in claims 1 to 14 comprising

- (a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, in antisense, sense or ambisense design,
- (b) followed by infection with an influenza carrier strain constructed to include flanking ribozyme target sequences in at least one of its viral RNA  
25 segments, and
- (c) thereafter selective vRNA inactivation through ribozyme cleavage.

16. A method of constructing influenza carrier strains carrying one or more ribozyme target sites (of type one) in vRNA flanking positions  
30 comprising

- (a) RNA polymerase I synthesis of recombinant vRNAs *in vivo*, carrying two different 3' promoter sequences in tandem, which are separated by a

second type of ribozyme target sequence, and which carry the said internal ribozyme target sites of type one;

(b) followed by infection of an influenza wildtype strain;

(c) thereafter amplification through simple steps of viral propagation; and

5 (d) finally isolation through removal of their external 3' promoter sequence by ribozyme cleavage through infection of cells expressing ribozyme type 2, followed by plaque purification.

10 17. A ribozyme-sensitive influenza carrier strain obtainable by the method of claim 16.

18. A pharmaceutical composition comprising a recombinant influenza virus according to claims 1 to 14.

15 19. Use of a recombinant influenza virus according to claims 1 to 14 for preparing a medicament for vaccination purposes.

20. The use according to claim 19, wherein the medicament

(a) is suitable against influenza and/or against other infections;

20 (b) is present in form of inactivated preparations; and/or

(c) is present in form of live recombinant viruses.

21. Use of a recombinant influenza virus according to claims 1 to 14 for preparing agents for somatic gene therapy.

25

22. Use of a recombinant influenza virus according to claims 1 to 14 for preparing agents, for transfer and expression of foreign genes into cells infected by such viruses.

30 23. Use of a recombinant influenza virus according to claims 1 to 14 for preparing agents for transfer and expression of RNA molecules into cells infected by such viruses.

24. The use of claim 23, wherein the RNA molecules to be expressed are antisense sequences or double-strand sequences relative to the target cell cellular mRNA molecules, and/or the agent is suitable for sequence-specific gene silencing, preferably by antisense RNA or RNA interference mechanisms.

25. The use according to claims 21 to 24, wherein the agents are applicable in *ex vivo* and *in vivo* application schemes.

26. A method for the production of proteins or glycoproteins which comprises utilizing a recombinant influenza virus according to claims 1 to 14 as expression vector.

27. The method of claim 26, wherein the production is performed in cell culture cells or in fertilized chicken eggs.

28. A method for preventing and/or treating influenza which comprises administering an effective amount of a recombinant influenza virus according to claims 1 to 14 to the mammal to be treated.

29. A method for somatic gene therapy, which method comprises ~~subjecting the organism to be treated with a recombinant influenza virus~~ according to claims 1 to 14.

30. A method for transfer and expression of foreign genes into cells, and for transfer and expression of RNA molecules into cells, which method comprises infecting the cells with a recombinant influenza virus according to claims 1 to 14.

31. Use of a recombinant influenza virus according to claims 1 to 14 for preparing agents for autologous immunotherapy.

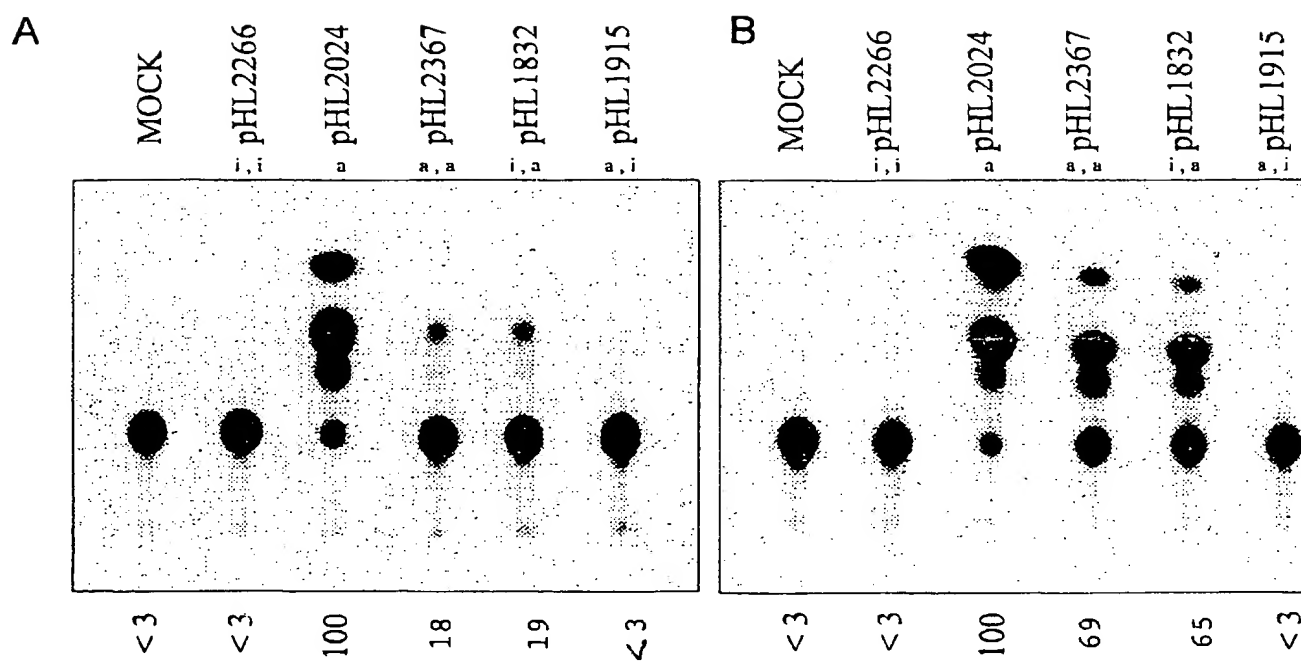


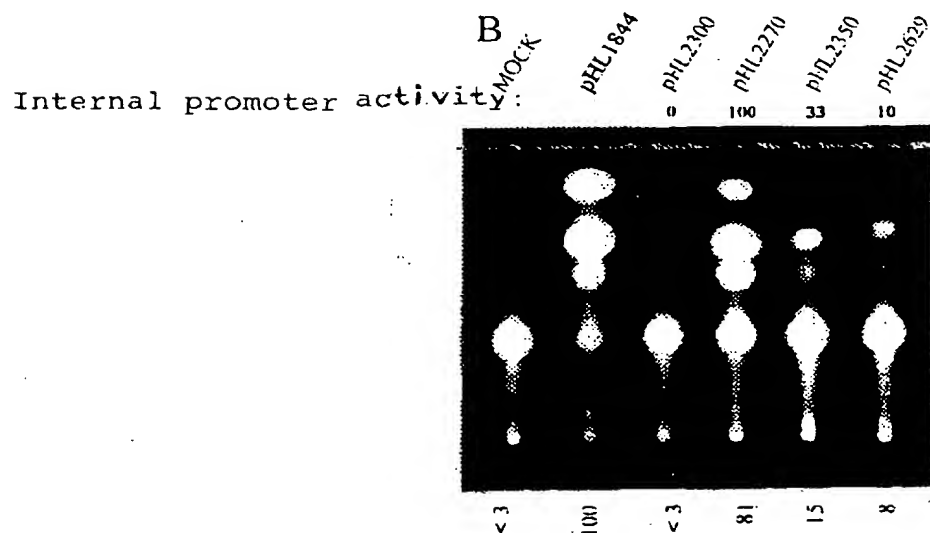
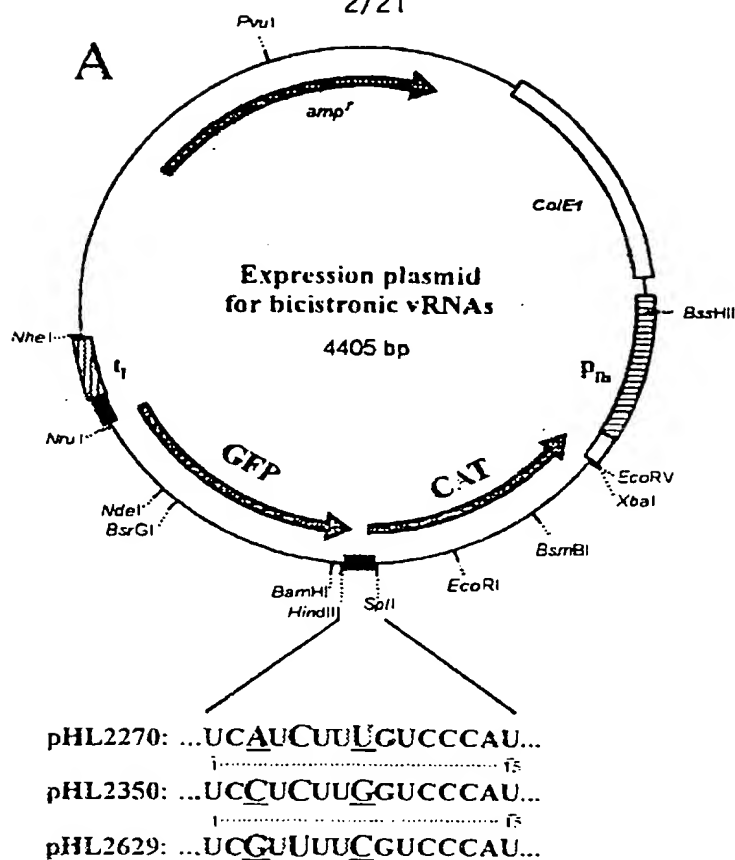
32. A method for an immunotherapy which comprises *ex vivo* infection of immune cells with a recombinant influenza virus according to claims 1 to 14, and introduction of the transduced cells into the patient.

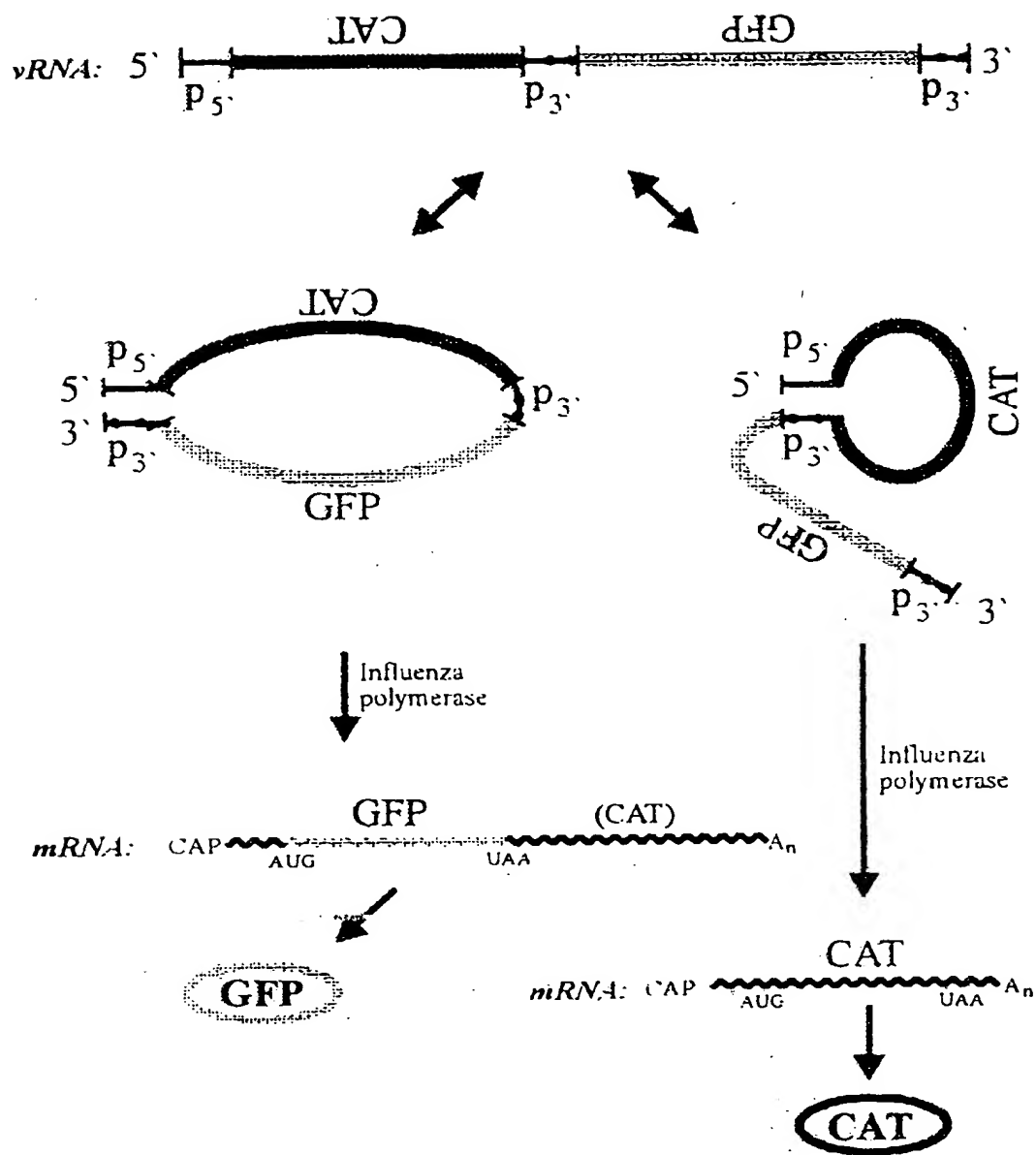
5

33. A method for the induction of antibodies which comprises utilizing a recombinant influenza virus according to claims 1 to 14 as an immunogen.

Fig. 1



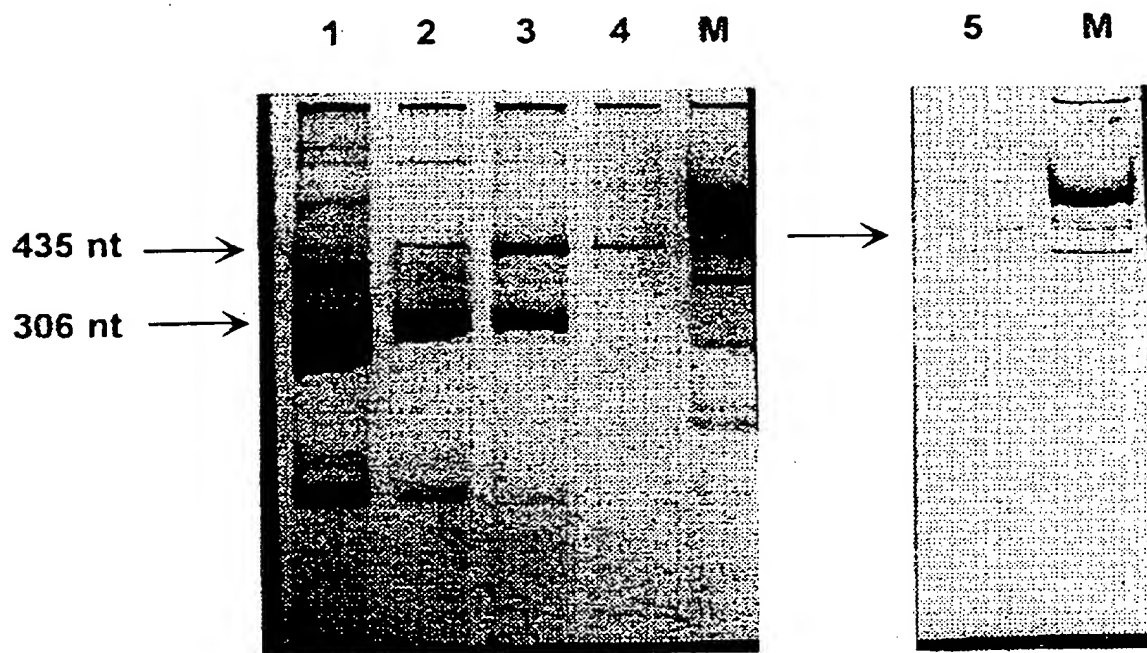


3/21  
FIG. 3

SUBSTITUTE SHEET (RULE 26)

4/21

FIG. 4



5/21

FIG. 5

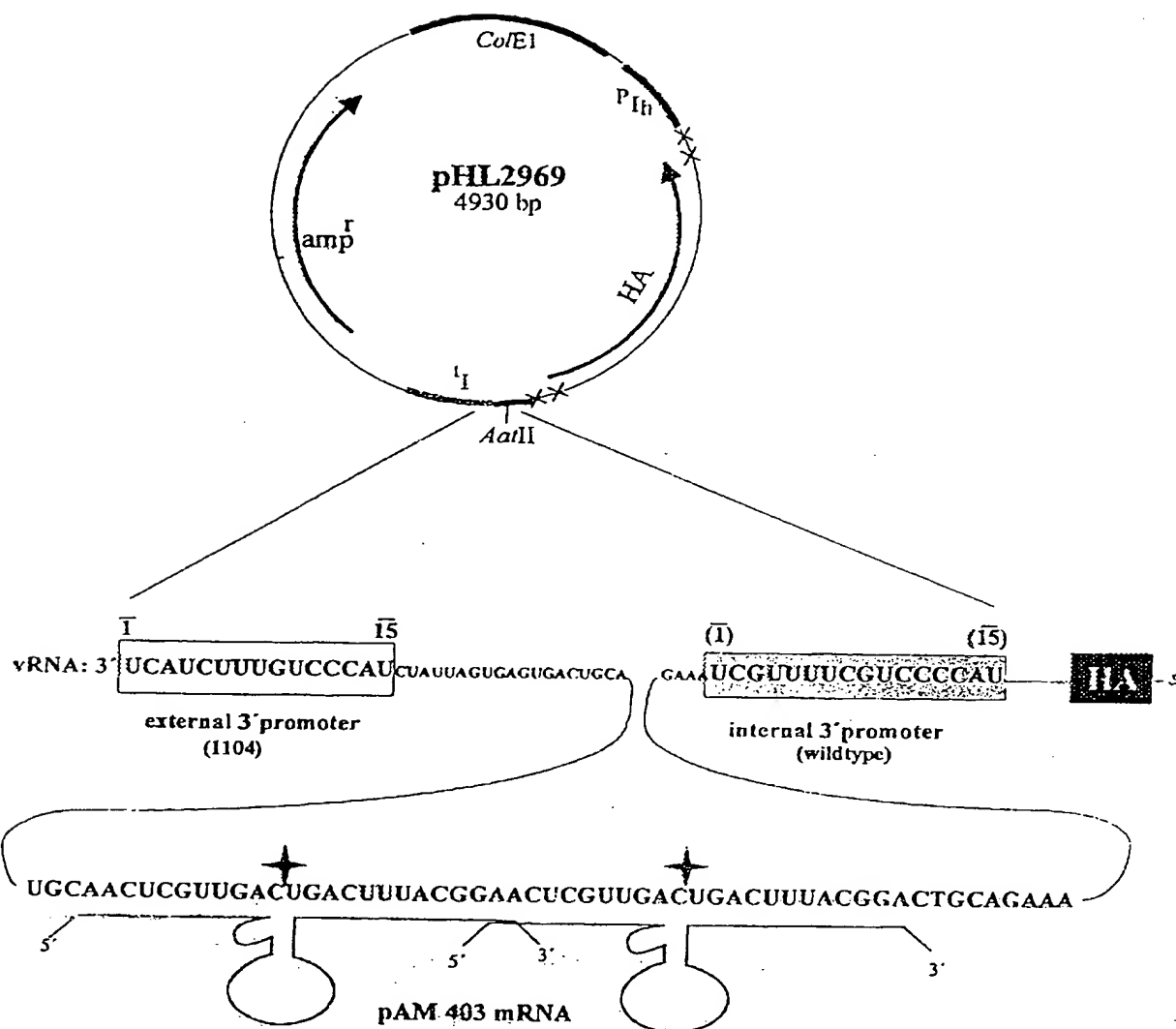


FIG. 6

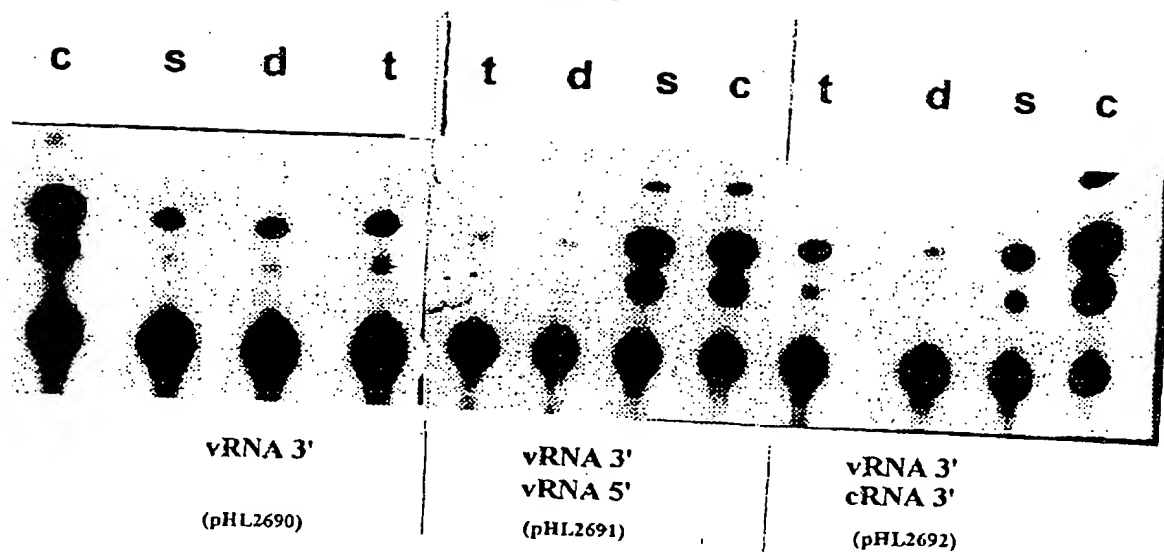
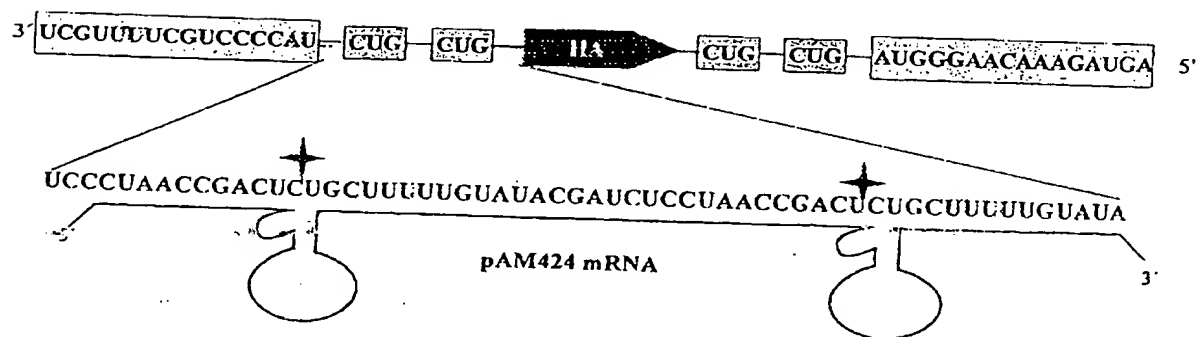
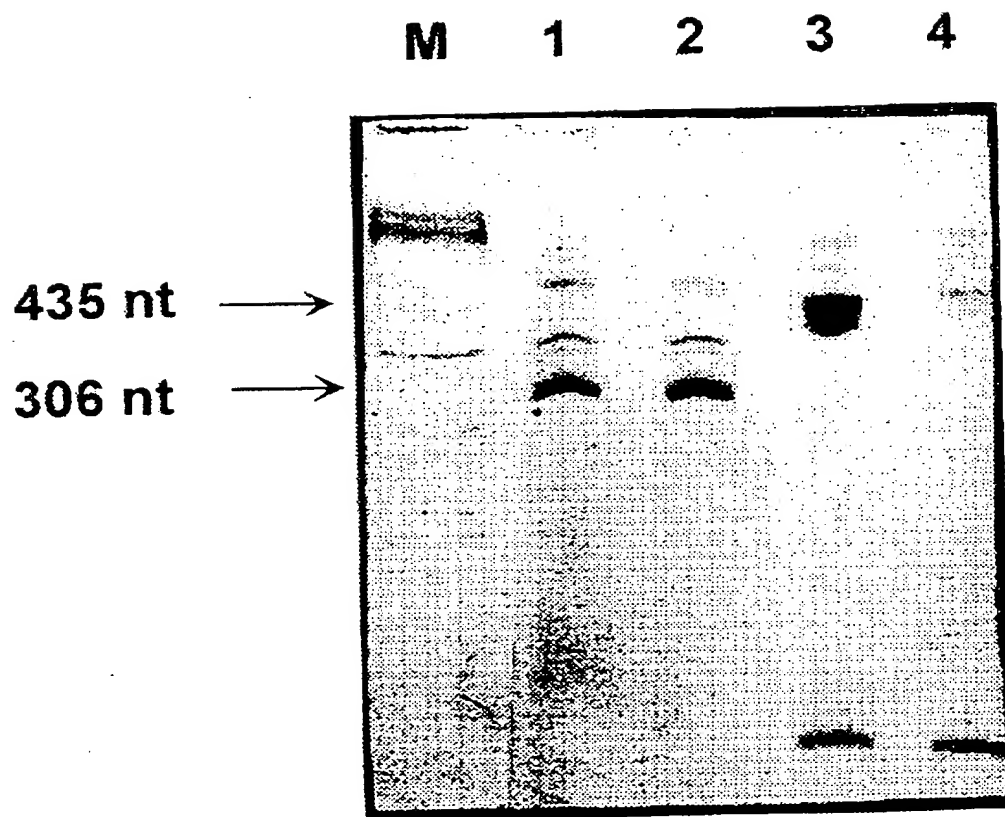


FIG. 7



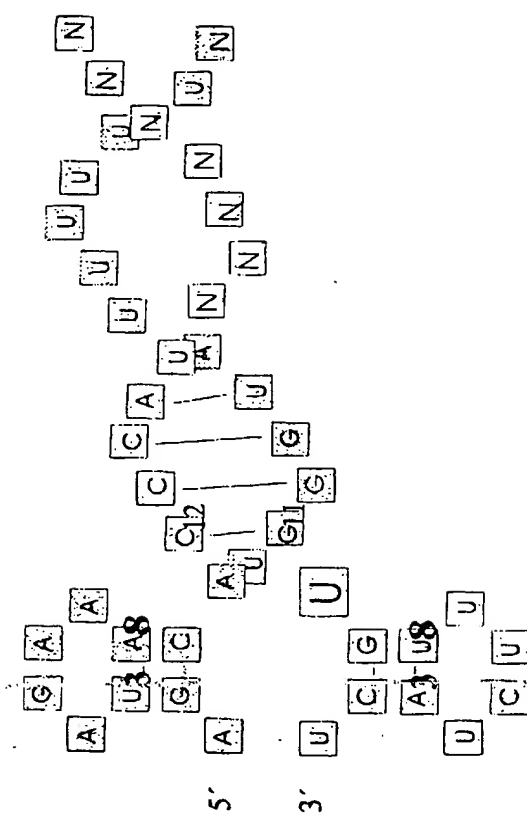
**FIG. 8**





8/21

FIG. 9



**SUBSTITUTE SHEET (RULE 26)**

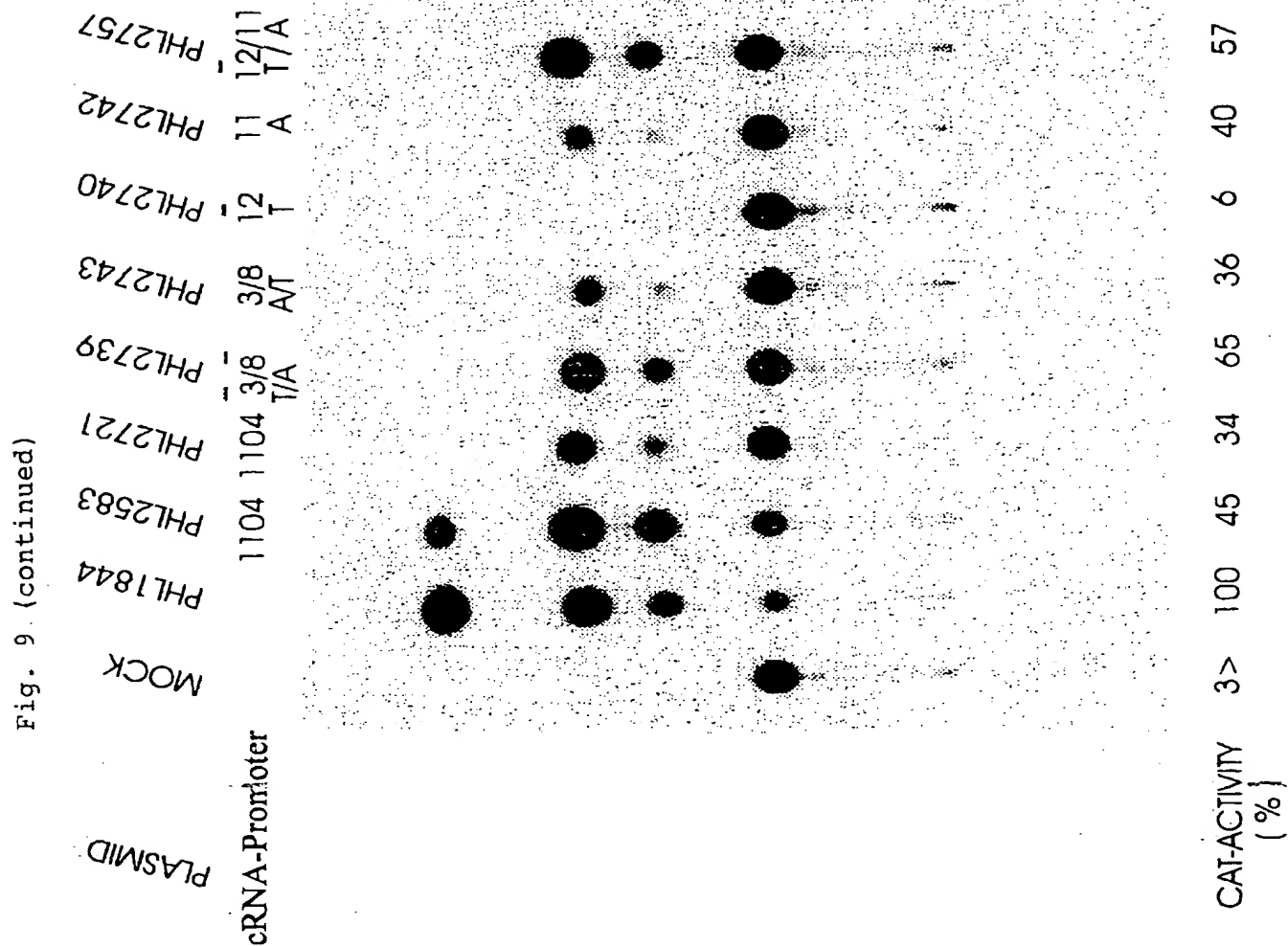


FIG. 10

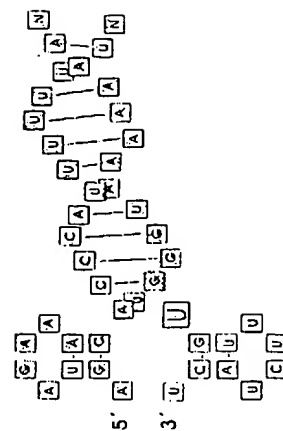
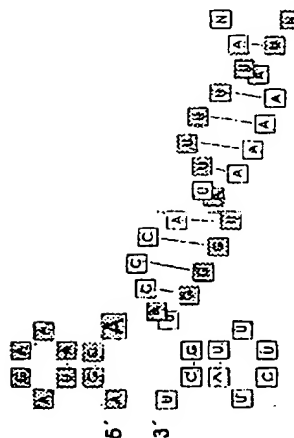
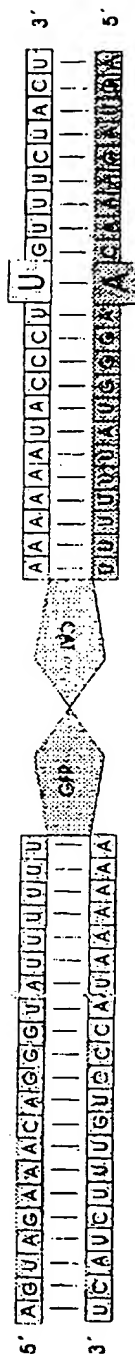
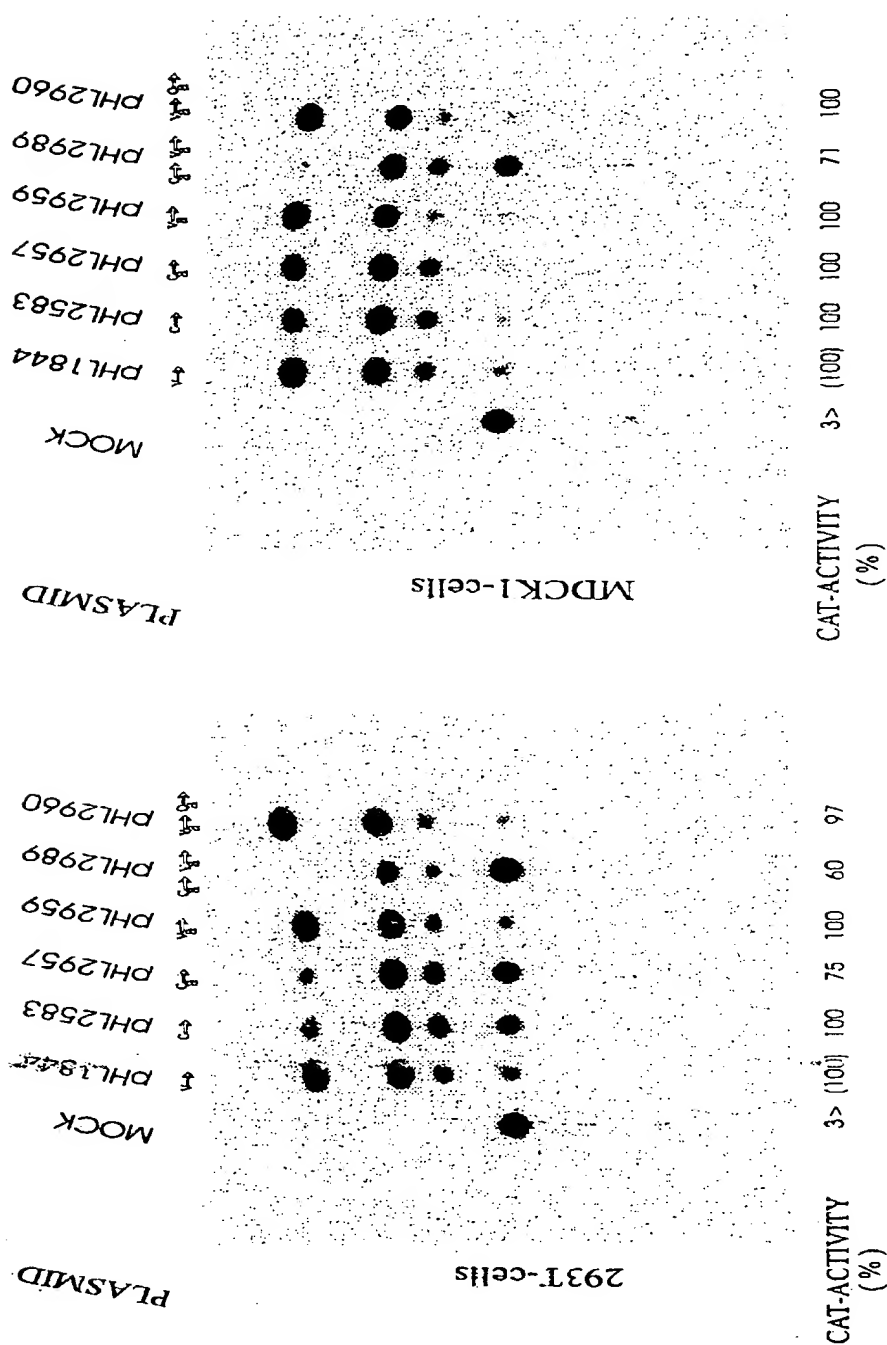
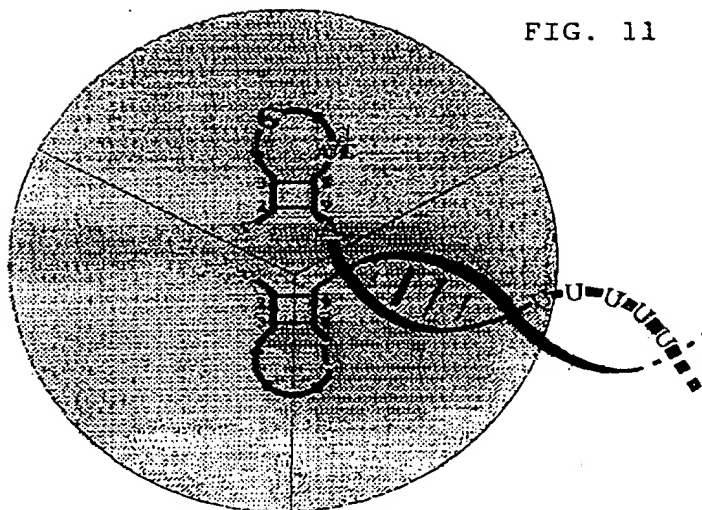


Fig. 10 (continued)



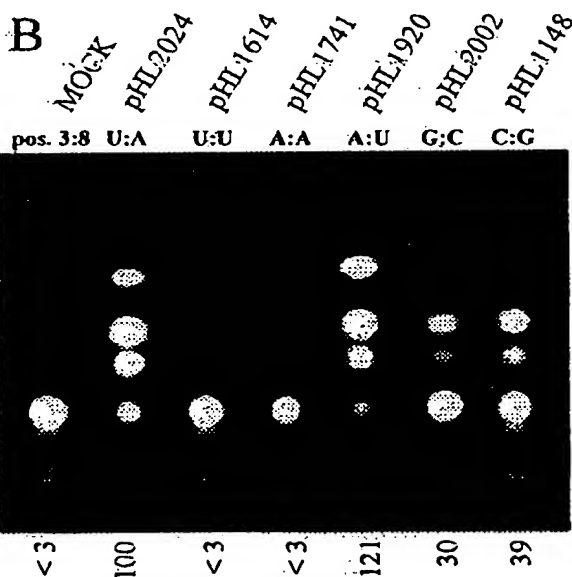
A

FIG. 11

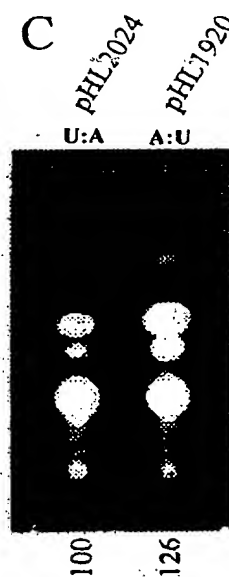


bp-variant position	G-C	A-U	C-G	U-A
2-5	pHL2024 100%	pHL1921 41%	pHL2003 < 3%	pHL2004 < 3%
3-8	pHL2002 36%	pHL1920 121%	pHL1148 63%	pHL2024 100%
2-9	pHL1945 11%	pHL1946 30%	pHL2024 100%	pHL1923 28%
3-8	pHL2428 6%	pHL2024 100%	pHL1948 33%	pHL1922 97%

B

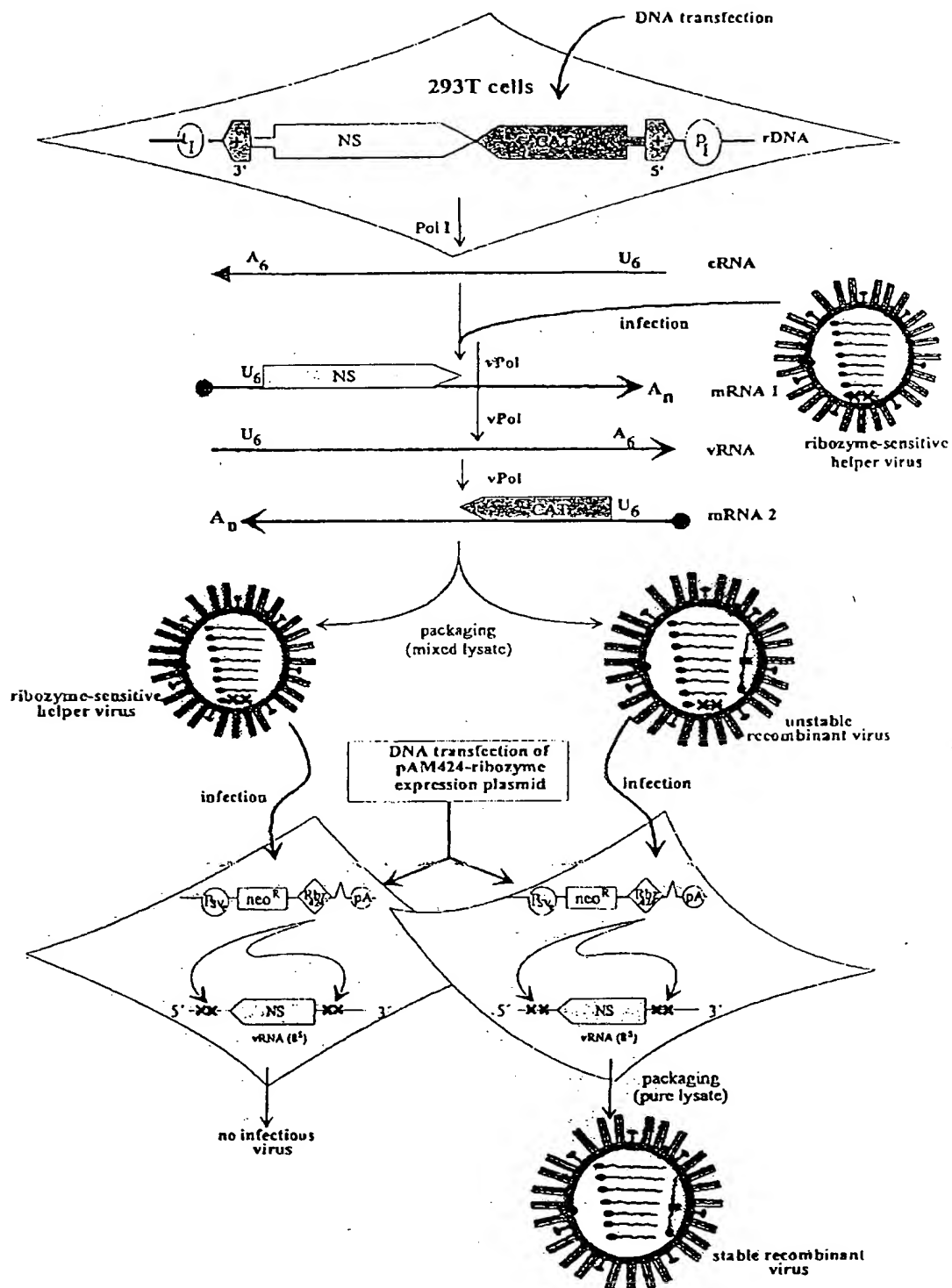


C



13/21

FIG. 12



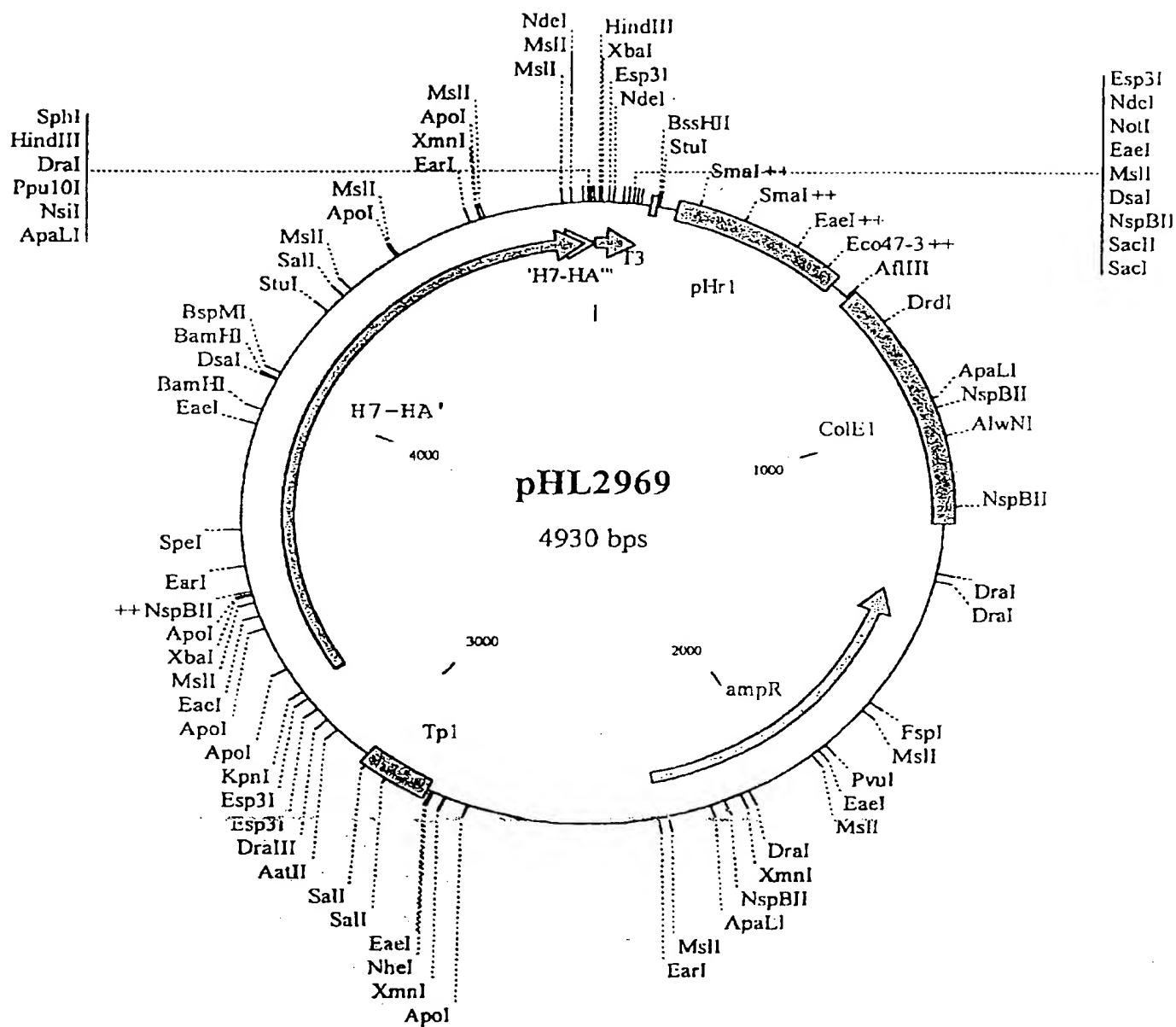
SUBSTITUTE SHEET (RULE 26)

14/21

FIG. 13



FIG. 14





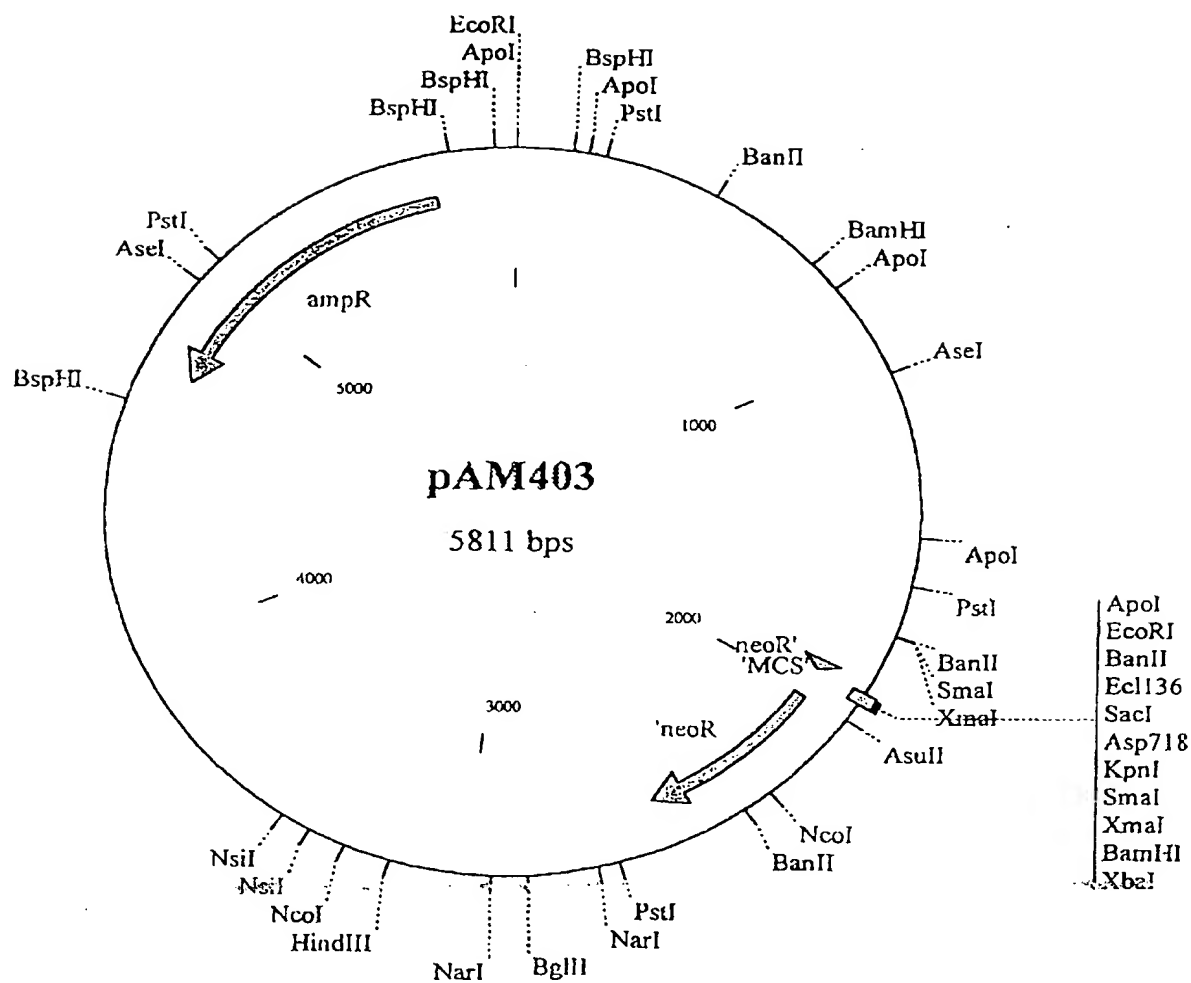
18/21  
FIG. 15

FIG. 16

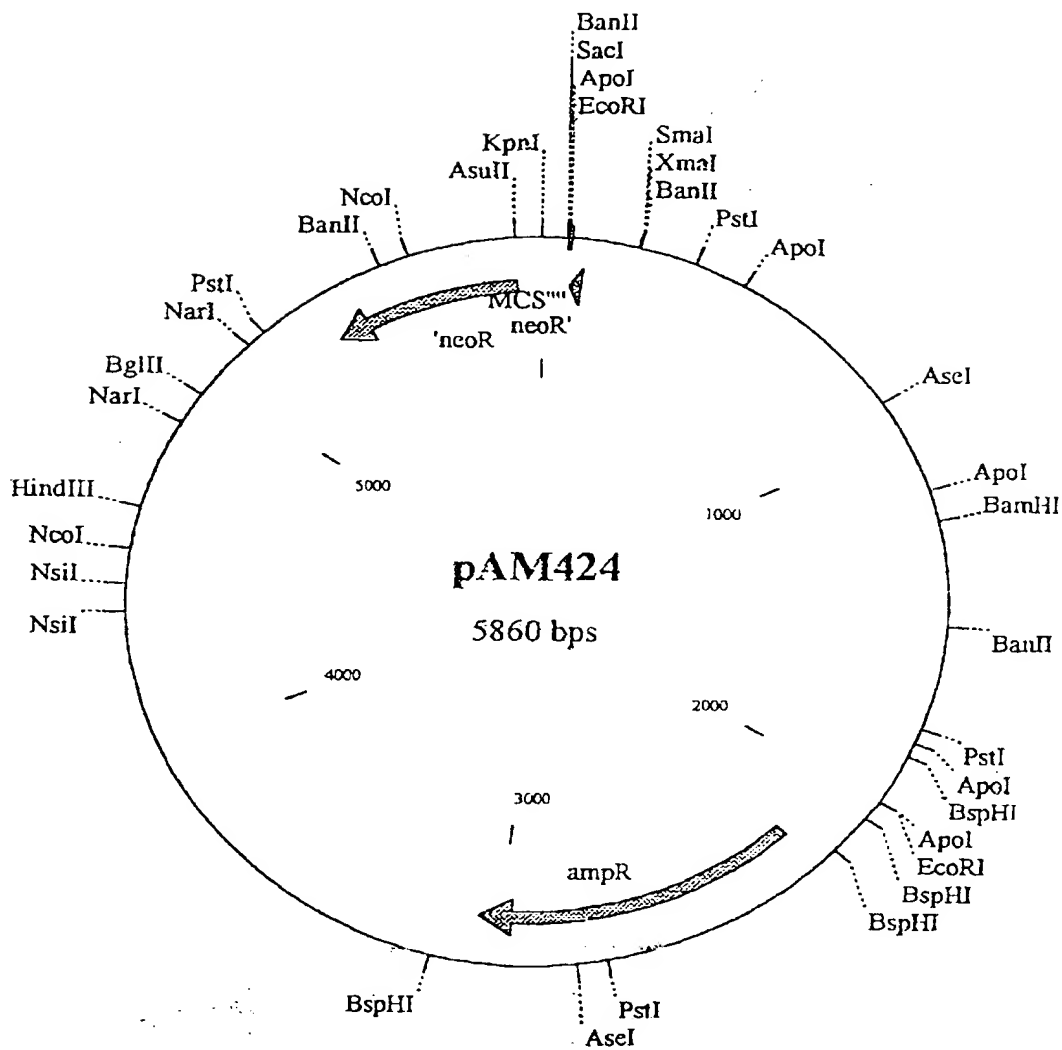


FIG. 17

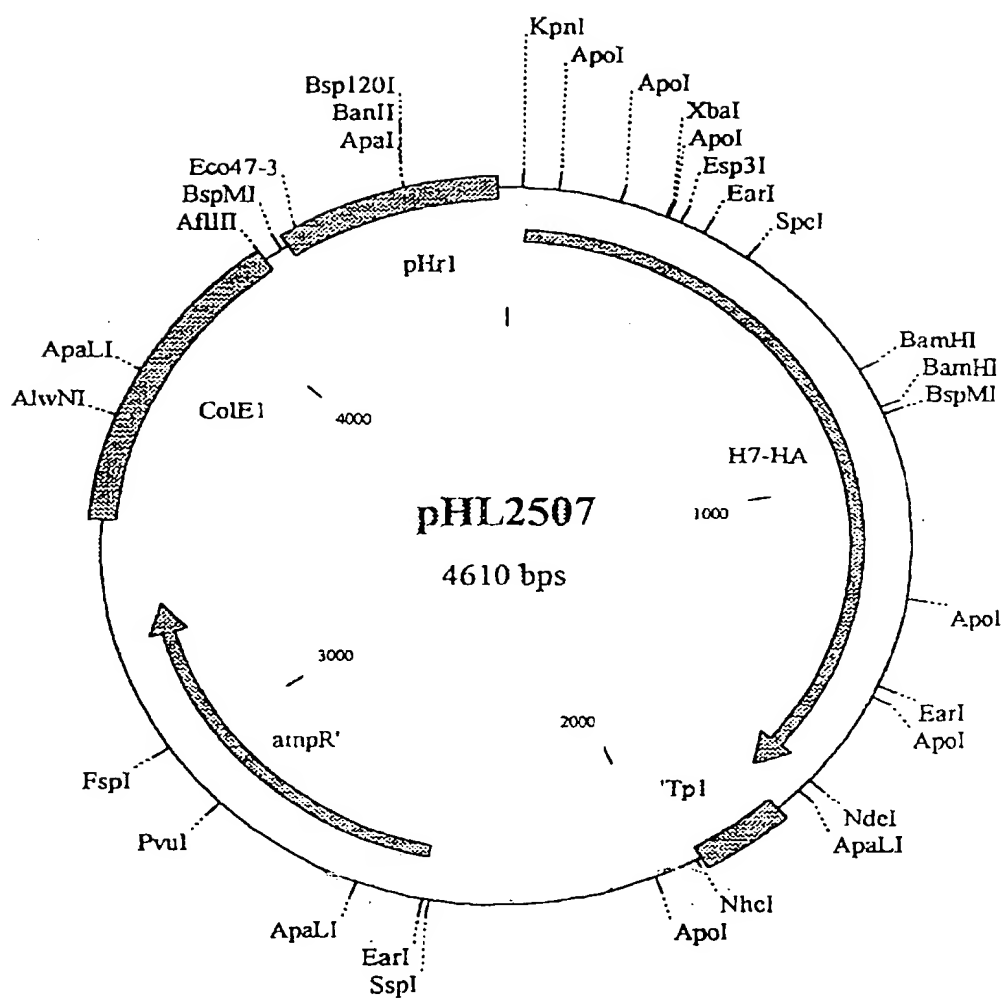
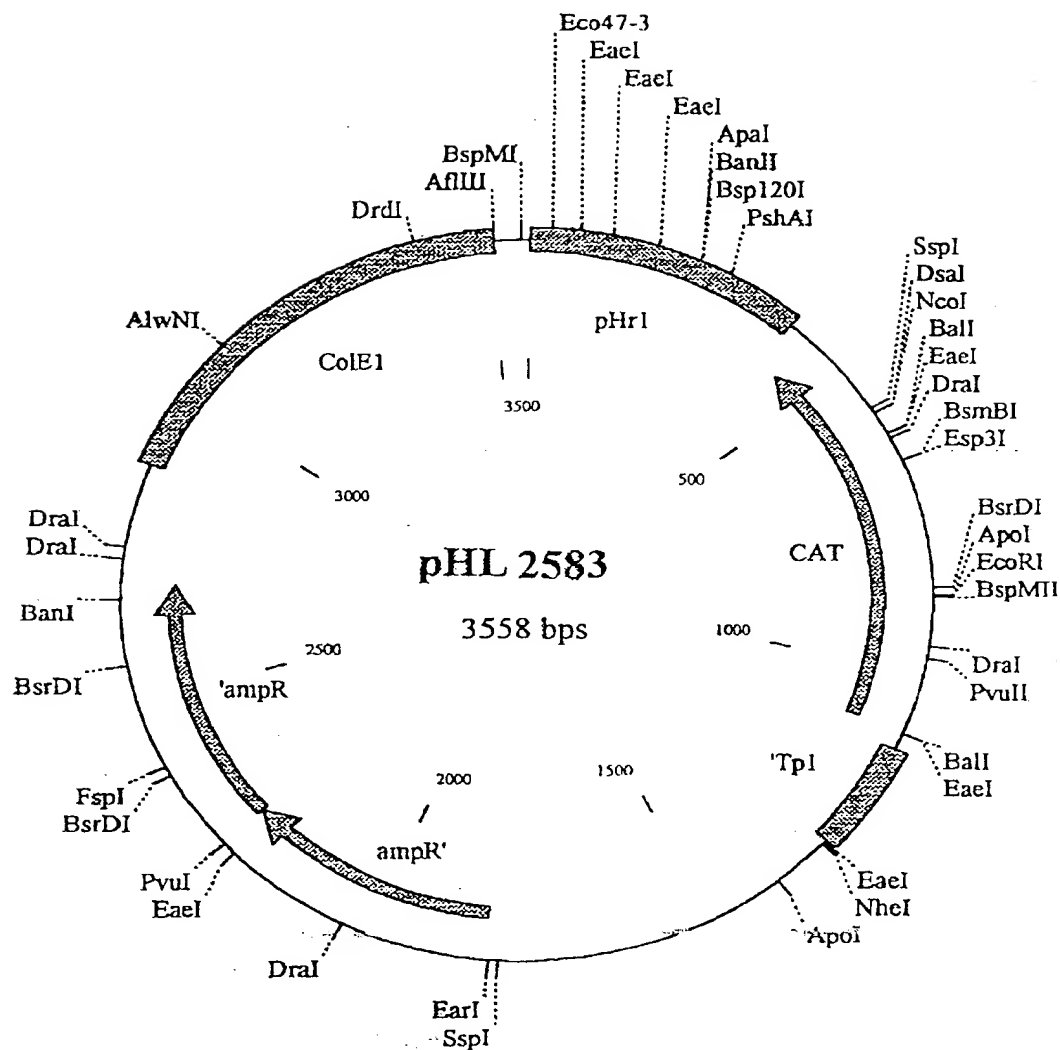
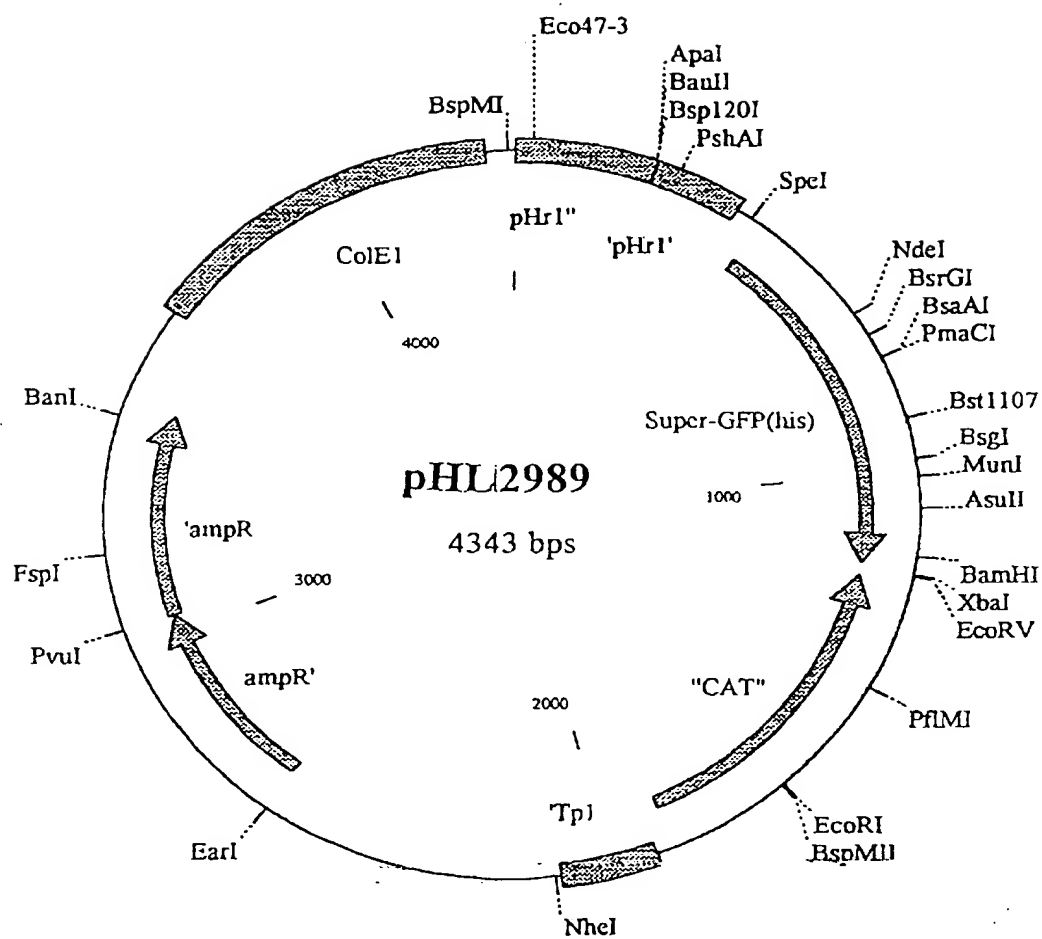
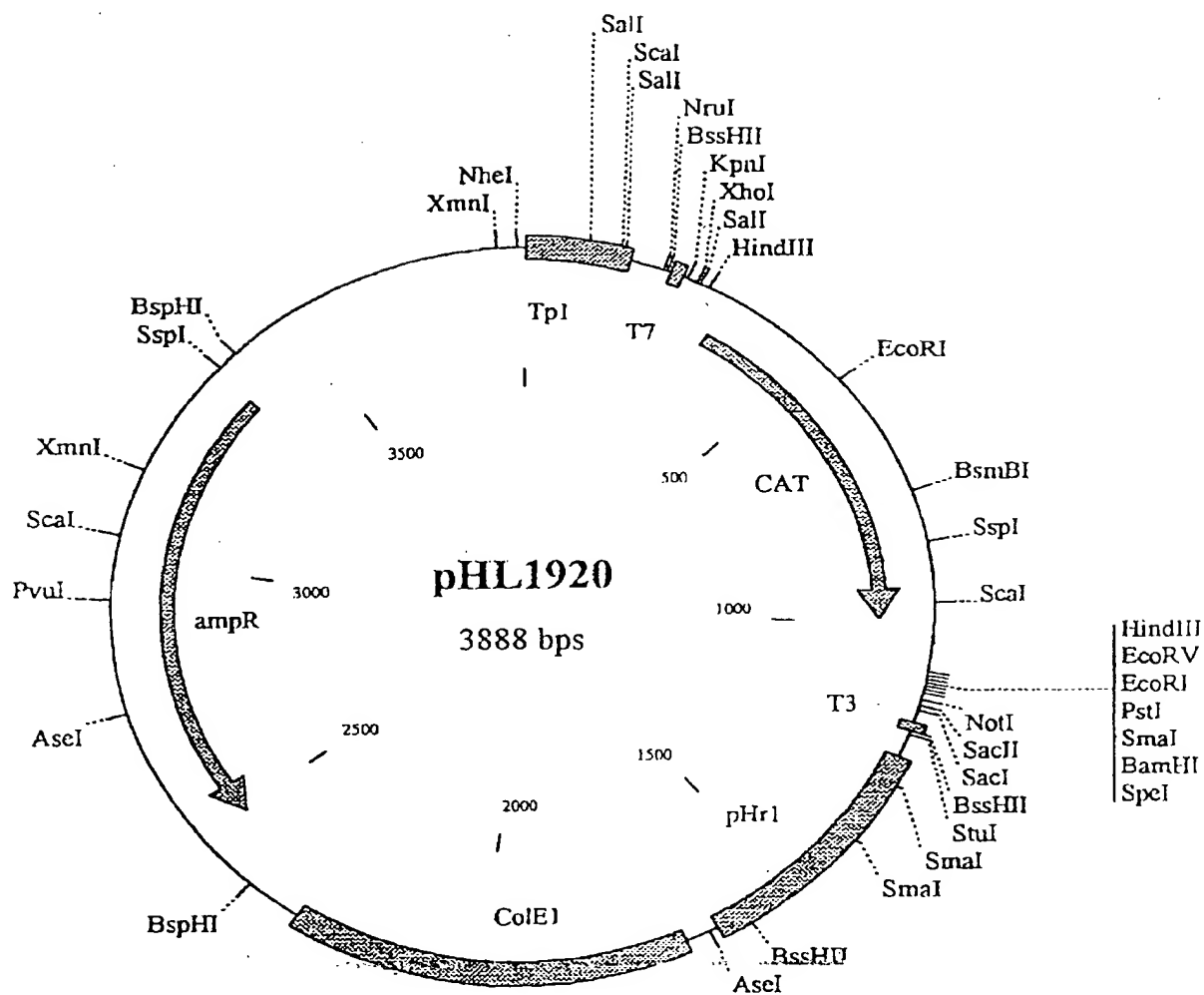


FIG. 18







## SEQUENCE LISTING

&lt;110&gt; ARTEMIS PHARMACEUTICALS GmbH

5 <120> Stable Recombinant Influenza Viruses Free of Helper  
Viruses

&lt;130&gt; 000520wo/JH/ml

10 <140>  
<141>

&lt;160&gt; 26

15 &lt;170&gt; PatentIn Ver. 2.1

&lt;210&gt; 1

&lt;211&gt; 4930

20 &lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: pHL2969

25

&lt;400&gt; 1

	cgtacgaagc	ttctagaggg	attggctgag	acgaaaaaca	tatgctagag	ggattggctg	60
	agacgaaaaa	catatgctag	agcggccgcc	accgcggtgg	agctccagct	ttgtttccct	120
	ttagtgaggg	ttaattgcgc	gcaggcctag	ctaggtaaaag	aaaaataccc	ttgtttctac	180
30	taataacccg	gcggcccaaa	atgccgactc	ggagcgaaaag	atatacctcc	cccggggccg	240
	ggagggtcgc	tcaccgacca	cgccgcccgc	ccaggcgacg	cgcgacacgg	acacctgtcc	300
	ccaaaaacgc	caccatcgca	gccacacacg	gagcgcccgg	ggccctctgg	tcaaccccag	360
	gacacacgcg	ggagcagcgc	cgggcccggg	acgccctccc	ggccgcccgt	gccacacgca	420
	ggggggccgg	ccgtgtctcc	agagcgggag	ccggaagcat	tttcggccgg	cccctcctac	480
35	gaccgggaca	cacgagggac	cgaaggccgg	ccaggcgcgca	cctctcgggc	cgcacgcgcg	540
	ctcagggagc	gctctccgac	tccgcacggg	gactcgccag	aaaggatcgt	gacctgcatt	600
	aatgaatacg	gggataacgc	aggaaagaac	atgtgagcaa	aaaggccagca	aaaggccagg	660
	aaccgtaaaa	aggccgcggt	gctggcggtt	ttccataggc	tccgcccccc	tgacgagcat	720
	cacaaaaatc	gacgctcaag	tcagaggtgg	cgaaaccgga	caggactata	aagataccag	780
40	gcgtttcccc	ctggaagctc	cctcgtgcgc	tctcctgttc	cgaccctgcc	gcttaccgga	840
	tacctgtccg	cctttctccc	ttcgggaagc	gtggcgcttt	ctcatagctc	acgctgtagg	900
	tatctcagtt	cggtgtaggt	cgttcgctcc	aagctgggct	gtgtgcacga	accccccggt	960
	cagcccgacc	gctgcgcctt	atccggtaac	tatcgtcttg	agtccaaccc	ggtaaagacac	1020
	gacttatcgc	cactgggaag	agccactggt	acagggatta	gcagagcgag	gtatgtagtc	1080
45	ggtgctacag	agttcttgaa	gtggtggcct	aactacggct	acactagaag	gacagtatct	1140
	ggtatctgcg	ctctgctgaa	gccagttacc	ttcggaaaaa	gagttggtag	ctcttgatcc	1200
	ggcaaaacaa	ccaccgctgg	tagcgggtgt	ttttttgttt	gcaagcagca	gattacgcgc	1260
	agaaaaaaag	gatctcaaga	agatcctttg	atcttttcta	cggggtctga	cgctcagtgg	1320
	aacgaaaact	cacgttaagg	gatttttggtc	atgagattat	caaaaaggat	cttcacctag	1380
50	atccttttaa	attaaaaatg	aagtttttaa	tcaatctaaa	gtatatatga	gtaaaacttg	1440
	tctgacagtt	accaatgctt	aatcagtgag	gcacctatct	cagcgatctg	tctatttcgt	1500
	tcattccatag	ttgcctgact	ccccgtcgtg	tagataacta	cgatacggga	ggccttacca	1560
	tctggcccca	gtgctgcaat	gataccgcga	gacccacgct	caccggctcc	agatttatca	1620
	gcaataaacc	agccagccgg	aaggggccgag	cgcagaagtg	gtcctgcaac	tttatccgcc	1680
55	tccatccagt	ctattaattg	ttgccgggaa	gctagagtaa	gtagtctgcc	agttaatatg	1740
	ttgcgcaacg	ttgttgccat	tgctacaggg	atcgtgggtg	cacgctcgtc	gtttgggtatg	1800
	gcttcattca	gctccgggtc	ccaacgatca	aggcgagtta	catgatcccc	catgttgtgc	1860
	aaaaaagcgg	ttagctcctt	cggctcctccg	atcgttgtca	gaagtaagtt	ggccgcagtg	1920
	ttactactca	tggttatggc	agcactgcac	aattctctta	ctgtcatgcc	atcgtgaagg	1980
60	tgctttttctg	tgaactgtga	gtactcaacc	aagtcattct	gagaatagtg	tatgcggcga	2040
	ccgagttgct	cttgcccggc	gtcaacacgg	gataataaccg	cgccacatag	cagaacttta	2100

5 aaagtgtctca tcattggaaa acgttcttcg gggcgaaaac tctcaaggat cttaccgctg 2160  
ttgagatcca gttcgatgta acccactcgt gcacccaact gatcttcagc atcttttact 2220  
ttcaccagcg tttctgggtg agcaaaaaca ggaaggcaaa atgccgcaaa aaagggataa 2280  
agggcgacac ggaaatggtg aatactcata ctcttccttt ttcaatatta ttgaagcatt 2340  
5 tatcagggtt attgtctcat gagcggatag atatttgaat gtatttagaa aaataaacia 2400  
aagagtttgt agaaacgcaa aaaggccatc cgtcaggatg gccttctgct taatttgatg 2460  
cctggcagtt tatggcgggc gtcctgcccg ccaccctccg ggccggttgc tgcgaacggt 2520  
caaatccgct cccggcggat ttgtcctact caggagagcg ttcaccgaca aacaacagat 2580  
aaaacgaaag gcccagtcct tgcactgagc ctttcgtttt atttgatgcc tggcagttcc 2640  
10 ctactctcgc atggggagac ccacactac catcggcgct acggcgtttc acttctgagt 2700  
tcggcatggg gtcagggtgg accacgcgc tactgccgac aggcataatc tgtttttatca 2760  
gaccgcttct cgttctctgat ttaatctgta tcaggctgaa aatcttctct catccgcaa 2820  
aacagaagct agcggccgat ccccaaaaaa aaaaaaaa aaaaaaaa gagtccagag 2880  
tggccccgcc gttccgcgcc gggggggggg gggggggggg acactttcgg acatctggtc 2940  
15 gacctccagc atcgggggaa aaaaaaaa caaagtctcg cccggagtac tggtcgacct 3000  
ccgaagtggg gggggagtag aaacagggtg gataatcact cactgacgta cgttgagcaa 3060  
ctgactgaaa tgccttgagc aactgactga aatgcctgac gtcttttagc aaagcagggt 3120  
agataatcac tcaactgagt acatccacat cgtaccagga ttggctgaga cgaaaaacat 3180  
atgtaccag ggattggctg agacgaaaaa catattgtag gtacaaaaat gaacactcaa 3240  
20 atcctggttt tcgcccttgc ggcagtcac cccacaaatg cagacaaaat ttgtcttggg 3300  
catcatgctg tatcaaatgg caccaaagta aacacactca ctgagagagg agtagaagtt 3360  
gtcaatgcaa cggaacagat ggagcggaca aacatcccca aaatttgctc aaaagggaaa 3420  
agaaccactg atcttggcca atgcggactg ttagggacca ttaccggacc acctcaatgc 3480  
gaccaatttc tagaattttc agctgatcta ataatcgaga gacgagaagg aaatgatgtt 3540  
25 tgttaccggg ggaagtgtgt taatgaagag gcattgcgac aaatcctcag aggatcagg 3600  
gggattgaca aagaaacaat gggattcaca tatagtggaa taaggaccaa cggaacaact 3660  
agtgcattga gaagatcagg gtcttcattc tatgcagaaa tggagtggct cctgtcaaat 3720  
acagacaatg cttctttccc acaaatgaca aaatcataca aaaacacagg gagagaatca 3780  
gctctgatag tctggggaat ccaccattca ggatcaacca ccgaacagac caaactatat 3840  
30 gggagtggaa ataaactgat aacagtcggg agttccaaat atcatcaatc ttttgtgccg 3900  
agtccaggaa cagcaccgca gataaatggc cggtcgggac ggattgattt tcattgggtg 3960  
atcttggatc ccaatgatac agttactttt agtttcaatg gggctttcat agtccaaat 4020  
cgtgccagct tcttgagggg aaagtccatg gggatccaga gcgatgtgca ggttgatgct 4080  
aattgcgaag gggaatgcta ccacagtggg gggactataa caagcagatt gccttttcaa 4140  
35 aacataaata gcagagcagt tggcaaatgc ccaagatatg taaaacagga aagtttatta 4200  
ttggcaactg ggatgaagaa cgttcccga ccttccaaaa aaaggaaaaa aagagggcctg 4260  
tttgggtgcta tagcagggtt tattgaaaat ggttgggaag gtctgggtcg cgggtgggtac 4320  
ggtttcaggc atcagaatgc acaaggagaa ggaactgcag cagactacaa aagcacccaa 4380  
tcggcaattg atcagataac cggaagttta aatagactca ttaagaaaaa caaccagcaa 4440  
40 tttgagctaa tagataatga attcactgaa gtggaaaagc agattggcaa ttttaattac 4500  
tggaccaaag actccatcac agaagtatgg tcttacaatg ctgaacttct tgtggcaatg 4560  
gaaaaccagc acactattga tttggctgat tcagagatga acaagctgta tgagcgagtg 4620  
aggaacaat taaggcaaaa tgctgaagag gatggcactg gttgctttga aatttttcat 4680  
aaatgtgacg atgattgtat ggctagtata aggaacaata cttatgatca cagcaaatat 4740  
45 agagaagaag cgatgcaaaa tagaatacaa attgacccag tcaaattgag tagtggctac 4800  
aaagatgtga tacttttggt tagcttcggg gcatcatgct ttttgcttct tgccattgca 4860  
atgggccttg ttttcatatg tgtgaagaac ggaaacatgc ggtgcactat atgcatttaa 4920  
agcttgcattg 4930

50

&lt;210&gt; 2

&lt;211&gt; 5811

&lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

55

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: pAM403

60

&lt;400&gt; 2

aatttcctttg cctaatttaa atgaggactt aacctgtgga aatattttga tgtgggaagc .60  
tgttactgtt aaaactgagg ttattggggg aactgctatg ttaaacttgc attcagggac 120



	acaaaaaact	catgaaaatg	gtgctggaaa	acccattcaa	gggtcaaatt	ttcatttttt	180
	tgctgttggt	ggggaacctt	tggagctgca	gggtgtgtta	gcaaactaca	ggaccaataa	240
	tcctgctcaa	actgtaaccc	caaaaaatgc	tacagttgac	agtcagcaga	tgaacactga	300
	ccacaaggct	gttttgata	aggataatgc	ttatccagtg	gagtgctggg	ttcctgatcc	360
5	aagtaaaaaat	gaaaacacta	gatatTTTTg	aacctacaca	gggtggggaaa	atgtgcctcc	420
	tgTTTTgcac	attactaaca	cagcaaccac	agtgtctctt	gatgagcagg	gtgttgggcc	480
	cttgtgcaaa	gctgacagct	tgtatgtttc	tgctgttgac	atttgtgggc	tgtttaccaa	540
	cacttctgga	acacagcagt	ggaagggaact	tcccagatat	tttaaaatta	cccttagaaa	600
	gcgggtctgtg	aaaaacccct	acccaatttc	ctttttgtta	agtgaacctaa	ttaacaggag	660
10	gacacagagg	gtggatgggc	agcctatgat	tggaatgtcc	tctcaagtag	aggaggttag	720
	ggTTTTatgag	gacacagagg	agcttcctgg	ggatccagac	atgataagat	acattgatga	780
	gtttggacaa	accacaacta	gaatgcagtg	aaaaaaatgc	tttatttgtg	aaatttgtga	840
	tgctattgct	ttatttgtaa	ccattataag	ctgcaataaa	caagttaaca	acaacaattg	900
	cattcatttt	atgtttcagg	ttcaggggga	gggtgtgggag	gtttttttaa	gcaagtaaaa	960
15	cctctatgaa	tgtggtatgg	ctgattatga	tctctagtca	aggcactata	catcaaatat	1020
	tccttataaa	cccctttaca	aattaaaaag	ctaaagggtac	acaatttttg	agcattagtt	1080
	ttaatagcag	acactctatg	cctgtgtgga	gtaagaaaaa	acagtatgtt	atgattataa	1140
	ctgttatgcc	tacttataaa	ggttacagaa	tatttttcca	taattttctt	gtatagcagt	1200
	gcagcttttt	cctttgtggt	gtaaatagca	aagcaagcaa	gagttctatt	actaaacaca	1260
20	gcatgactca	aaaaacttag	caattctgaa	ggaaagtcc	tgggggtctt	tacctttctc	1320
	ttcttttttg	gaggagtaga	atgttgagag	tcagcagtag	cctcatcatc	actagatggc	1380
	atcttctctg	agcaaaacag	gttttctctc	ttaaaggcat	tccaccactg	ctcccatcca	1440
	tcagttccat	aggttggaa	ctaaaataca	caaacaatta	gaatcagtag	tttaacacat	1500
	tatacactta	aaaattttat	atttacctta	gagctttaaa	tctctgtagg	tagtttgtcc	1560
25	aattatgtca	caccacagaa	gtaagggtcc	ttcacaagaa	tccgggacca	aagcgcccat	1620
	cgtgcctccc	cactcctgca	gttcgggggc	atggatgcgc	ggatagccgc	tgctggtttc	1680
	ctggatgccg	acggatttgc	actgccggta	gaactccgcg	aggtcgtcca	gcctcaggca	1740
	gcagctgaac	caactcgcga	ggggatcgag	cccgggggtg	gcgaagaact	ccagcatgag	1800
	atccccgcgc	tggaggatca	tccagccggc	gtcccggaaa	acgattccga	agcccaacct	1860
30	ttcatagaag	gcggcggtgg	aatcgaaatc	tcgtgatggc	aggttgggcg	tcgcttggtc	1920
	ggtcatttctg	atgaattcga	gctcggtacc	cggggatcct	ctagaggcat	ttcagtttctg	1980
	tcctcacgga	ctcatcagag	ttgtctcaat	cgaaccccag	agtcccgcctc	agaagaactc	2040
	gtcaagaagg	cgatagaagg	cgatgcgctg	cgaatcggga	gcggcgatac	cgtaaagcac	2100
	gaggaagcgg	tcagcccatt	cgccgcctaag	ctcttcagca	atatcacggg	tagccaacgc	2160
35	tatgtcctga	tagcgggtccg	ccacaccccag	ccggccacag	tcgatgaatc	cagaaaagcg	2220
	gccattttcc	accatgatat	tcggcaagca	ggcatcgcca	tgggtcacga	cgagatcctc	2280
	gccctcgggc	atgcgcgcct	tgagcctggc	gaacagttcg	gctggcgcgga	gcccctgatg	2340
	ctctctcgcc	agatcatcct	gatcgacaag	accggcttcc	atccgagtag	gtgctcgctc	2400
	gatgcgatgt	ttcgcttggt	ggtcgaatgg	gcaggtagcc	ggatcaagcg	tatgcagccg	2460
40	ccgcatttga	tcagccatga	tggatacttt	ctcggcagga	gcaagggtgag	atgacaggag	2520
	atcctgcccc	ggcacttcgc	ccaatagcag	ccagtccctt	cccgttctag	tgacaacgtc	2580
	gagcacagct	gcgcaaggaa	cgcccgctct	ggccagccac	gatagccgcg	ctgcctcgctc	2640
	ctgcagttca	ttcagggcac	cggacaggtc	ggtcttgaca	aaaagaaccg	ggcgcccttg	2700
	cgctgacagc	cggaacacgg	cggcatcaga	gcagccgatt	gtctgtgtgt	ccagttcaat	2760
45	gccgaatagc	ctctccaccc	aagcggcccg	agaacctgcg	tgcaatccat	cttgttcaat	2820
	catgcgaaac	gatactcatc	ctgtctcttg	atcagatctt	gatccccctgc	gccatcagat	2880
	ccttggcggc	aagaaagcca	tccagtttac	tttgcagggc	ttcccaacct	taccagaggg	2940
	cgccccagct	ggcaattccg	gttcgcttgc	tgtccataaa	accgcccagt	ctagctatcg	3000
	ccatgtaagc	ccactgcaag	ctacctgctt	tctctttgcg	cttgcgtttt	cccttgtcca	3060
50	gatagcccag	tagctgacat	tcataccggg	tcagcacctg	ttctgcggac	tggcttttcta	3120
	cgtgttccgc	ttccttttagc	agcccttgcg	ccttgagtgc	ttgcggcagc	gtgaagcttt	3180
	ttgcaaaaagc	ctaggcctcc	aaaaaagcct	cctcactact	tctggaatag	ctcagaggcc	3240
	gaggcggcct	cggcctctgc	ataaaataaaa	aaaatttagtc	agccatgggg	cggagaatgg	3300
	gcggaactgg	gcggaggttag	ggcggggatg	ggcgaggtta	ggggcgggac	tatgggttgc	3360
55	gactaattga	gatgcagctg	ttgcatactt	ctgcctgctg	gggagcctgg	ggactttcca	3420
	cacctggttg	ctgactaatt	gagatgcag	ctttgcatac	ttctgcctgc	tggggagcct	3480
	ggggactttc	cacaccctaa	ctgacacaca	ttccacagct	gcctcgcgcg	tttcggtgat	3540
	gacggtgaaa	acctctgaca	catgcagctc	ccggagacgg	tcacagcttg	tctgtaagcg	3600
	gatgccggga	gcagacaagc	cctgcagggc	gcgtcagcgg	gtgttggcgg	gtgtcggggc	3660
60	gcagccatga	cccagtcacg	tcgcatgagc	gcagtgtata	ctggcttaac	tatgcggcat	3720
	cagagcagat	tgtactgaga	gtgcaccata	tgcggtgtga	aataccgcac	agatgcgtaa	3780

5 ggagaaaata ccgcatcagg cgctcttccg ctctctcgct cactgactcg ctgcgctcgg 3840  
tcgttcggct gcggcgagcg gtatcagctc actcaaaggc ggtaatacgg ttatccacag 3900  
aatcagggga taacgcagga aagaacatgt gagcaaaagg ccagcaaaag gccagggaacc 3960  
gtaaaaaggc cgcgttgctg gcgtttttcc ataggctcgg cccccctgac gagcatcaca 4020  
aaaatcgacg ctcaagtcag aggtggcgaa acccgacagg actataaaga taccaggcgt 4080  
ttccccctgg aagctccctc gtgcgctctc ctgttccgac cctgccgctt accggatacc 4140  
tgtccgcctt tctcccttcg ggaagcgtgg cgctttctca tagctcacgc tgtagggtatc 4200  
tcagttcggt gtaggtcggt cgctccaagc tgggctgtgt gcacgaaccc cccgttcagc 4260  
ccgaccgctg cgccttatcc ggtaactatc gtcttgagtc caaccggta agacacgact 4320  
10 tatcgccact ggcagcagcc actggtaaca ggattagcag agcgaggat gtaggcgggtg 4380  
ctacagagtt cttgaagtgg tggcctaact acggctacac tagaaggaca gtatttggtgta 4440  
tctgcgctct gctgaagcca gttaccttcg gaaaaagagt tggtagctct tgatccggca 4500  
aacaaccac cgctggtagc ggtgggtttt ttgtttgcaa gcagcagatt acgcgcagaa 4560  
aaaaaggatc tcaagaagat cctttgatct tttctacggg gtctgacgct cagtggaaacg 4620  
15 aaaactcacg ttaagggatt ttggtcatga gattatcaaa aaggatcttc acctagatcc 4680  
ttttaaatta aaaatgaagt tttaaatcaa tctaaagtat atatgagtaa acttgggtctg 4740  
acagttacca atgcttaatc agtgaggcac ctatctcagc gatctgtcta tttcgttcat 4800  
ccatagttgc ctgactcccc gtcgtgtaga taactacgat acgggagggc ttaccatctg 4860  
gccccagtg cgaatgata ccgcgagacc cacgctcacc ggctccagat ttatcagcaa 4920  
20 taaaccagcc agccggaagg gccgagcgca gaagtggtec tgcaacttta tccgcctcca 4980  
tccagtcctat taattgttgc cgggaagcta gagtaagtag ttcgccagtt aatagtttgc 5040  
gcaacgttgt tgccattgct gcaggcatcg tgggtgtcac ctcgtcggtt ggtatggctt 5100  
cattcagctc cggttcccaa cgatcaaggc gagttacatg atcccccatg ttgtgcaaaa 5160  
aagcggttag ctcttcggt cctccgatcg ttgtcagaag taagttggcc gcagtgttat 5220  
25 cactcatggt tatggcagca ctgcataatt ctcttactgt catgccatcc gtaagatgct 5280  
tttctgtgac tggtagtac tcaaccaagt cattctgaga atagtgtatg cggcgaccga 5340  
gttgctcttg cccggcgta acacgggata ataccgcgcc acatagcaga actttaaaaag 5400  
tgctcatcat tggaaaacgt tcttcggggc gaaaactctc aaggatctta ccgctgttga 5460  
gatccagttc gatgtaaccc actcgtgcac ccaactgatc ttcagcatct tttactttca 5520  
30 ccagcgtttc tgggtgagca aaaacaggaa ggcaaaatgc cgcaaaaaag ggaataaggg 5580  
cgacacggaa atgttgaaata ctcatactct tcctttttca atattattga agcatttatc 5640  
agggttattg tctcatgagc ggatacatat ttgaattgat ttgaaaaaat aaacaaatag 5700  
gggttcgcg caccattccc cgaaaagtgc cacctgacgt ctaagaaacc attattatca 5760  
35 tgacattaac ctataaaaat aggcgtatca cgaggccctt tcgtcttcaa g 5811

<210> 3

<211> 2005

<212> DNA

40 <213> Artificial Sequence

<220>

<223> Description of Artificial Sequence: vHM41

45 <400> 3

agtagaaaca agggatatttt tctttaccta gctaggcctg cgcgcaatta accctcacta 60  
aagggaaaca aagctggagc tccaccgcgg tggcgccgc tctagcatat gtttttcgtc 120  
tcagccaatc cctctagcat atgtttttcg tctcagccaa tccctctaga agcttcgtac 180  
gcatgcaagc tttaaatgca tatagtgcac cgcatgtttc cgttcttcac acatagtaaa 240  
50 acaaggccca ttgcaatggc aagaagcaaa aagcatgatg ccccgaaagc aaaccaaagt 300  
atcacatctt tgtagccact actcaatttg actgggtcaa tttgtattct attttgcac 360  
gcttcttctc tgtatttgcg gtgatcataa gtattgttcc ttatactagc catacaatca 420  
tcgtcacatt tatgaaaaat ttcaaagcaa ccagtgccat cctcttcagc attttccctt 480  
aattgttttc tcactcgctc atacagcttg ttcattctcg aatcagccaa atcaaatagt 540  
55 tgcgtgtttt ccattggcac agaaagtcca gtcattgtaag accatacttc tgtgatggag 600  
tctttgggtc agttaattaa attgccaatc tgcttttcca cttcagtgaa ttcattatct 660  
attagctcaa attgctgggt ggttttctta atgagtctat ttaactttcc gggtatctga 720  
tcaattgccg attgggtgct tttgtagtct gctgcagttc cttctccttg tgcattctga 780  
tgccatgaac cgtaccaccc gtcgaccaga ccttcccaac cattttcaat aaaccctgct 840  
60 atagcaccaa acaggcctct ttttttcctt tttttggaag gttcgggaac gttcttctac 900  
ccagttgccca ataataaact ttcctggttt acatatcttg ggcatttgcc aactgctctg 960

ctatattatgt tttgaaaagg caatctgctt gttatagtcc ctccactgtg gtagcattcc 1020  
 ccttcgcaat tagcatcaac ctgcacatcg ctctggatcc ccattggactt tcccctcaag 1080  
 aagctggcac gatttggagc tatgaaagcc ccattgaaac taaaagtaac tgtatcattg 1140  
 ggatccaaga tcaaccaatg aaaatcaatc cgtccggacc ggccatttat ctgcggtcgt 1200  
 5 gttcctggac tcggcacaaa agattgatga tatttggaac tcccgaactgt tatcagttta 1260  
 tttccactcc catatagttt ggtctgttcg gtggttgatc ctgaatggtg gattccccag 1320  
 actatcagag ctgattctct ccctgtgttt ttgtatgatt ttgtcatttg tgggaaagaa 1380  
 gcattgtctg tatttgacag gagccactcc atttctgcat agaatgaaga ccctgatctt 1440  
 ctacatgcac tagttgttcc gttggtcctt attccactat atgtgaatcc cattgtttct 1500  
 10 ttgtcaatcc cacctgatcc tctgaggatt gtgcgcaatg cctcttcatt aacaaacttc 1560  
 cccgggtaac aaacatcatt tccctctcgt ctctcgatta ttagatcagc tgaaaattct 1620  
 agaaattggt cgcattgagg tgggtccggt atgggtcccta acagtccgca ttggccaaga 1680  
 tcagtggttc ttttcccttt tgagcaaatt ttggggatgt ttgtccgctc cactgtttcc 1740  
 gttgcattga caacttctac tcctctctca gtgagtgtgt ttactttggt gccatttgat 1800  
 15 acagcatgat gtccaagaca aattttgtct gcatttgtgg ggatgactgc cgcaagggcg 1860  
 aaaaccagga tttgagtgtt cattttggta cctacaatat gtttttcgtc tcagccaatc 1920  
 cctggtacaa tatgtttttc gtctcagcca atcctggtac gatgtggatg tcactcagtg 1980  
 agtgattatc taccctgctt ttgct 2005

20

<210> 4  
 <211> 1146  
 <212> DNA  
 <213> Artificial Sequence

25

<220>  
 <223> Description of Artificial Sequence: vHM81

<400> 4  
 30 agtagaaaca agggatatttt tctttaccta gctaggcctg cgcgcaatta accctcacta 60  
 aagggaacaa aagctggagc tccaccgcgg tggcgccgcg tctagcatat gtttttcgtc 120  
 tcagccaate cctctagcat atgtttttcg tctcagccaa tccctctaga agcttcgtac 180  
 gcatgcttaa ataagctgaa acgagaaagt tcttatctct tgctccactt caagcggtag 240  
 ttgtaaggct tgcataaatg ttatttgttc aaaactattc tctgttatct tcaatctatg 300  
 35 tctcactttc tcaattaacc atcttatttc ttcaaatttc tgactcaatt gttctcgcca 360  
 ttttccgttt ctgctttgga gggagtggag gtcccccatt ctcttactct cttctccaag 420  
 cgaatctctg tatagtttca gagactcgaa ctgtgttata attccattca agtccctcga 480  
 tgaggacccc aattgcattt ttgacatcct catcagtatg tcctggaaga gaaggcaatg 540  
 gtgaaatttc gccgacaatt gctccctcat cgggttaaagc ccttaatagt atgagagttt 600  
 40 ccagccgata gaaaatcaca ctgaagtttg ctttcagtat gatgttcttc cccatgatcg 660  
 cctgggtccat tctgatgcaa agggagcctg ccactttctg tttgggcatg agcatgaacc 720  
 agtcccttga catctcttca agagtcagt cagttaggtg gcgtgtagca ggtacagagg 780  
 caatgggtcat tttaagtgcc tcatcggatt cgtccctcag aatccgctcc actatctgct 840  
 ttccaacacg agtagctgtg tccatgttca gaccaagagt gctgcctctt cccctcaggg 900  
 45 acttctgata tcggcgaagt cgggtcaagga atggggcatc acccatttct tggctctgca 960  
 atcgttttgc gacatgccaa agaaagcagt ctacctgaaa gcttgacaca gtgttggaat 1020  
 ccattatggt acctacaata tgtttttcgt ctcagccaat ccctggtaca atatgttttt 1080  
 cgtctcagcc aatcctggta cgatgtggat gtcactcagt gagtgattat ctaccctgct 1140  
 tttgct 1146

50

<210> 5  
 <211> 5860  
 <212> DNA  
 <213> Artificial Sequence

55

<220>  
 <223> Description of Artificial Sequence: pAM424

60

<400> 5  
 catcgattgg ctgactgatg agtccgtgag gacgaaacga aaaacatatt gtagagctcg 60

	aattcatcga	aatgaccgac	caagcgacgc	ccaacctgcc	atcacgagat	ttcgattcca	120
	ccgccgcctt	ctatgaaagg	ttgggcttcg	gaatcgtttt	ccgggacgcc	ggctggatga	180
	tcctccagcg	cggggatctc	atgctggagt	tcttcgcccc	ccccgggctc	gatccccctg	240
	cgagttgggt	cagctgctgc	ctgaggctgg	acgacctcgc	ggagttctac	cggcagtgca	300
5	aatccgtcgg	catccaggaa	accagcagcg	gctatccgcg	catccatgcc	cccgaactgc	360
	aggagtgggg	aggcacgatg	gccgcttttg	tcccggatct	ttgtgaagga	accttacttc	420
	tgtggtgtga	cataattgga	caaactacct	acagagattt	aaagctctaa	ggtaaataata	480
	aaatttttaa	gtgtataatg	tgttaaacta	ctgattctaa	ttgttttgtt	attttagatt	540
	ccaacctatg	gaactgatga	atgggagcag	tgggtggaatg	cctttaatga	ggaaaacctg	600
10	ttttgtctag	aagaaatgcc	atctagttag	gatgaggcta	ctgctgactc	tcaacattct	660
	actcctccaa	aaaagaagag	aaaggtagaa	gaccccaagg	actttccttc	agaattgcta	720
	agttttttga	gtcatgctgt	gttttagtaat	agaactcttg	cttgctttgc	tatttacacc	780
	acaaaggaaa	aagctgcact	gctatacaag	aaaattatgg	aaaaatattc	tgtaaccttt	840
	ataagtaggc	ataacagtta	taatcataac	atactgtttt	ttcttactcc	acacaggcat	900
15	agagtgtctg	ctattaataa	ctatgctcaa	aaattgtgta	ccttttagctt	tttaatttgt	960
	aaaggggtta	ataaggaata	tttgatgtat	agtgccttga	ctagagatca	taatcagcca	1020
	taccacattt	gtagaggttt	tacttgcttt	aaaaaacctc	ccacacctcc	ccctgaacct	1080
	gaaacataaa	atgaatgcaa	ttgttgttgt	taacttgttt	attgcagctt	ataatggtta	1140
	caataaaagc	aatagcatca	caaatttcac	aaataaagca	tttttttcac	tgcattctag	1200
20	ttgtggtttg	tccaaactca	tcaatgtatc	ttatcatgtc	tggatcccca	ggaagctcct	1260
	ctgtgtcctc	ataaacctta	acctcctcta	cttgagagga	cattccaatc	ataggctgcc	1320
	catccacctt	ctgtgtcctc	ctgttaatta	ggctacttaa	caaaaaggaa	attgggtagg	1380
	ggtttttcac	agaccgcttt	ctaagggtaa	ttttaaaata	tctgggaagt	cccttccact	1440
	gctgtgttcc	agaagtgttg	gtaaacagcc	cacaaatgca	aacagcagaa	acatacaagc	1500
25	tgtcagcttt	gcacaagggc	ccaacacctt	gctcatcaag	aagcactgtg	gttgctgtgt	1560
	tagtaatgtg	caaaaacagga	ggcacatttt	ccccacctgt	gtaggttcca	aaatatctag	1620
	tgttttcatt	tttacttgga	tcaggaaccc	agcactccac	tggataagca	ttatccttat	1680
	ccaaaacagc	cttgtgtgca	gtgttcatct	gctgactgtc	aactgtagca	ttttttgggg	1740
	ttacagtttg	agcaggatat	ttggtcctgt	agtttgctaa	cacaccttgc	agctccaaag	1800
30	gttccccacc	aacagcaaaa	aaatgaaaat	ttgaccttg	aatgggtttt	ccagcaccat	1860
	tttcatgagt	tttttgtgtc	cctgaatgca	agtttaacat	agcagttacc	ccaataacct	1920
	cagttttaac	agtaacagct	tcccacatca	aaatttttcc	acaggttaag	tcctcattta	1980
	aattaggcaa	aggaattctt	gaagacgaaa	gggcctcgtg	atacgccctat	ttttataggt	2040
	taatgtcatg	ataataatgg	tttcttagac	gtcaggtggc	acttttcggg	gaaatgtgcg	2100
35	cggaaacctt	atttgtttat	ttttctaaat	acattcaaat	atgtatccgc	tcatgagaca	2160
	ataaccctga	taaatgtctc	aataatattg	aaaaaggaag	agtatgagta	ttcaacattt	2220
	ccgtgtcgcc	cttattccct	tttttgccgc	attttgccct	cctgtttttg	ctcaccacga	2280
	aacgctgggt	aaagtaaaag	atgctgaaga	tcagttgggt	gcacgagtgg	gttacatcga	2340
40	actggatctc	aacagcggta	agatccttga	gagttttcgc	cccgaagaac	gttttccaat	2400
	gatgagcact	tttaaagtct	tgctatgtgg	cggcggtatta	tcccggtgtg	acgccgggca	2460
	agagcaactc	ggtcgccgca	tacactatct	tcagaatgac	ttgggtgagt	actcaccagt	2520
	cacagaaaag	catcttacgg	atggcatgac	agtaagagaa	ttatgcagtg	ctgccataac	2580
	catgagtgat	aacactgcgg	ccaacttact	tctgacaacg	atcggaggac	cgaaggagct	2640
	aaccgctttt	ttgcacaaca	tgggggatca	tgtaactcgc	cttgatcggt	gggaaccgga	2700
45	gctgaatgaa	gccataccaa	acgacgagcg	tgacaccacg	atgcctgcag	caatgggcaac	2760
	aacgttgccg	aaactattaa	ctggcgaact	acttactcta	gcttcccgcc	aacaattaat	2820
	agactggatg	gaggcggata	aagttgcagg	accacttctg	cgetccggcc	ttccggctgg	2880
	ctggtttatt	gctgataaat	ctggagccgg	tgagcgtggg	tctcgcggta	tcattgcagc	2940
	actggggcca	gatggtgaag	cctcccgtat	cgtagttatc	tacacgacgg	ggagtccagg	3000
50	aactatggat	gaacgaaata	gacagatcgc	tgagataggt	gcctcactga	ttaagcattg	3060
	gtaactgtca	gaccaagttt	actcatatat	acttttagatt	gattttaaacc	ttcattttta	3120
	atttaaaagg	atctaggtga	agatcctttt	tgataatctc	atgaccaaaa	tcccttaacg	3180
	tgagttttcg	ttccactgag	cgtcagaccc	cgtagaaaaag	atcaaaggat	cttcttgaga	3240
	tccttttttt	ctgcgcgtaa	tctgctgctt	gcaaacaaaa	aaaccaccgc	taccagcggg	3300
55	ggttttgttg	cgggatcaag	agctaccaac	tgtttttccg	aaggtaactg	gcttcagcag	3360
	agcgcagata	ccaaatactg	tccttctagt	gtagccgtag	ttaggccacc	acttcaagaa	3420
	ctctgtagea	ccgectacat	acctcgctct	gctaactcct	ttaccagtgg	ctgctgccag	3480
	tggcgataag	tcgtgtctta	ccgggttgga	ctcaagacga	tagttaccgg	ataaggcgca	3540
	gcggtcgggc	tgaacggggg	gttcgtgcac	acagcccagc	ttggagcgaa	cgacctacac	3600
60	cgaactgaga	tacctacagc	gtgagctatg	agaaagcgcc	acgcttcccg	aagggagaaa	3660
	ggcggacagg	tatccggtaa	gcggcagggg	cggaaacagga	gagcgcacga	gggagcttcc	3720

5 agggggaaac gcctggtatc tttatagtcc tgtcgggttt cgccacctct gacttgagcg 3780  
tcgatttttg tgatgctcgt cagggggggcg gagcctatgg aaaaacgcca gcaacgcggc 3840  
cttttttacgg ttcctggcct tttgctggcc ttttgctcac atgttctttc ctgcgttatc 3900  
ccctgattct gtggataacc gtattaccgc ctttgagtga gctgataccg ctgcgcgcag 3960  
ccgaacgacc gagcgcagcg agtcagttag cgaggaagcg gaagagcgcc tgatgcggta 4020  
ttttctcctt acgcctctgt gcggtatttc acaccgcata tgggtgactc tcagtacaat 4080  
ctgctctgat gccgcatagt taagccagta tacactccgc tatcgctacg tgactgggtc 4140  
atggctgcgc cccgacaccc gccaacaccc gctgacgcgc cctgacgggc ttgtctgctc 4200  
ccggcatccg cttacagaca agctgtgacc gtctccggga gctgcatgtg tcagaggttt 4260  
10 tcaccgtcat caccgaaacg cgcgaggcag ctgtggaatg tgtgtcagtt aggggtgtgga 4320  
aagtccccag gctccccagc aggcagaagt atgcaaagca tgcattctcaa ttagtcagca 4380  
accagggtgtg gaaagtcccc aggtccccc gcaggcagaa gtatgcaaag catgcatctc 4440  
aattagtcag caaccatagt cccgccccta actccgcccc tcccggccct aactccgccc 4500  
agttccgccc attctccgcc ccattggtga taatttttt ttattttatgc agaggccgag 4560  
15 gccgcctcgg cctctgagct attccagaag tagtgaggag gcttttttgg aggcctaggc 4620  
ttttgcaaaa agcttcacgc tgccgcaagc actcaggggc caagggtctg taaaggaagc 4680  
ggaacacgta gaaagccagt ccgcagaaac ggtgctgacc ccggatgaat gtcagctact 4740  
gggctatctg gacaagggaa aacgcaagcg caaagagaaa gcaggtagct tgcagtggtc 4800  
ttacatggcg atagctagac tgggcgggtt tatggacagc aagcgaaccg gaattgccag 4860  
20 ctggggcgcc ctctggtaag gttgggaagc cctgcaaagt aaactggatg gctttcttgc 4920  
cgccaaggtat ctgatggcgc aggggatcaa gatctgatca agagacagga tgaggatcgt 4980  
ttcgcatgat tgaacaagat ggattgcacg caggttctcc ggccgcttgg gtggagaggc 5040  
tattcggcta tgactgggca caacagacaa tcggctgctc tgatgccgcc gtgttccggc 5100  
tgtcagcgca ggggcgccc gttctttttg tcaagaccga cctgtccggt gccctgaatg 5160  
25 aactgcagga cgaggcagcg cggctatcgt ggctggccac gacgggcgtt ccttgccgag 5220  
ctgtgctcga cgttgctact gaagcgggaa gggactggct gctattgggc gaagtgccg 5280  
ggcaggatct cctgtcatct caccttgctc ctgccagaaa agtatccatc atggctgatg 5340  
caatgcggcg gctgcatacg cttgatccgg ctacctgcc attcgaccac caagcgaaac 5400  
atcgatcga gcagcacgt actcggatgg aagccggtct tgtcgatcag gatgacttg 5460  
30 acgaagagca tcaggggctc gcgccagcgc aactgttcgc caggctcaag gcgcgcatg 5520  
ccgacggcga ggatctcgtc gtgacccatg gcgatgcctg cttgccgaat atcatggtg 5580  
aaaatggccg cttttctgga ttcctcgact gtggccggt ggggtgtggc gaccgctatc 5640  
aggacatagc gttggctacc cgtgatattg ctgaagagct tggcggcgaa tgggctgacc 5700  
gcttctcgt gctttacggt atcgccgctc ccgattcgca gcgcataccc ttctatcgcc 5760  
35 ttcttgacga gttcttctga gcgggactct ggggttcgaa tcctaccagg gattggctga 5820  
ctgatgagtc cgtgaggacg aaacgaaaaa catatggtac 5860

40 <210> 6  
<211> 4610  
<212> DNA  
<213> Artificial Sequence

45 <220>  
<223> Description of Artificial Sequence: pHL2507

50 <400> 6  
gaggcatttc agtcagttgc tcaaggtacc aaaatgaaca ctcaaatacct ggttttcgcc 60  
cttgccggcag tcatccccac aaatgcagac aaaatttgtc ttggacatca tgctgtatca 120  
aatggcacca aagtaaacac actcactgag agaggagtag aagttgtcaa tgcaacggaa 180  
acagtggagc ggacaaacat ccccaaaatt tgctcaaaag ggaaaagaac cactgatctt 240  
ggccaatgcg gactgttagg gaccattacc ggaccacctc aatgcgacca atttctagaa 300  
ttttcagctg atctaataat cgagagacga gaaggaaatg atgtttgtta cccggggaag 360  
tttggttaatg aagaggcatt gcgacaaatc ctgagaggat caggtgggat tgacaaagaa 420  
55 acaatgggat tcacatatag tggaaataagg accaacggaa caactagtgc atgtagaaga 480  
tcagggtctt cattctatgc agaaatggag tggctcctgt caaatacaga caatgcttct 540  
ttcccacaaa tgacaaaatc atacaaaaac acagggagag aatcagctct gatagtcttg 600  
ggaatccacc attcaggatc aaccaccgaa cagaccaaac tatatgggag tggaaaataa 660  
ctgataacag tcgggagttc caaatatcat caatcttttg tgccgagtc aggaacacga 720  
60 ccgcagataa atggccggtc cggacggatt gattttcatt ggttgatctt ggatcccaat 780  
gatacagtta cttttagttt caatggggct ttcatagctc caaatcgtgc cagcttcttg 840

	aggggaaaagt	ccatgggggat	ccagagcgat	gtgcaggttg	atgctaattg	cgaaggggaa	900
	tgctaccaca	gtggagggac	tataacaagc	agattgcctt	ttcaaaacat	aaatagcaga	960
	gcagttggca	aatgcccaag	atatgtaaaa	caggaaaagt	tattattggc	aactgggatg	1020
	aagaacgttc	ccgaaccttc	caaaaaaagg	aaaaaaaagag	gcctgttttg	tgctatagca	1080
5	gggttttattg	aaaatggttg	ggaaggtctg	gtcgacgggt	ggtacggttt	caggcatcag	1140
	aatgcacaag	gagaaggaac	tcagcagac	tacaaaagca	cccaatcggc	aattgatcag	1200
	ataaccggaa	agttaaatag	actcattaag	aaaaccaacc	agcaatttga	gctaatagat	1260
	aatgaattca	ctgaagtggg	aaagcagatt	ggcaatttaa	ttaactggac	caaagactcc	1320
	atcacagaag	tatggtctta	caatgctgaa	cttcttggtg	caatggaaaa	ccagcacact	1380
10	attgattttgg	ctgattcaga	gatgaacaag	ctgtatgagc	gagtggaggaa	acaattaagg	1440
	gaaaatgctg	aagaggatgg	cactgggttg	tttgaaattt	ttcataaatg	tgacgatgat	1500
	tgtatggcta	gtataaggaa	caatacttat	gatcacagca	aatacagaga	agaagcgatg	1560
	caaaatagaa	tacaaattga	cccagtcaaa	ttgagttagt	gctacaaaaga	tgtgatactt	1620
	tggttttagct	tcggggcatc	atgctttttg	cttcttgcca	ttgcaatggg	ccttgttttt	1680
15	atatgtgtga	agaacggaaa	catgcggtgc	actatttgta	tataggtttg	gaaaaaaaca	1740
	ccccctgttt	ctactcccc	ccaacttcgg	aggctcgacca	gtactccggg	cgaaactttt	1800
	tttttttttt	ttcccccgat	gctggaggtc	gaccagatgt	ccgaaagtgt	ccccccccc	1860
	ccccccccc	ggcgcggaac	ggcggggcca	ctctggactc	tttttttttt	tttttttttt	1920
	ttttttgggg	atcgggcgct	agcttctggt	ttggcggtat	agagaagatt	ttcagcctga	1980
20	tacagattaa	atcagaacgc	agaagcggtc	tgataaaaaca	gaatttgcc	ggcggcagta	2040
	gcgcggtggt	cccacctgac	cccatacgga	actcagaagt	gaaacgcctg	agcgccgatg	2100
	gtagtgtggg	gtctccccat	gcgagagttag	ggaactgcca	ggcatcaaat	aaaacgaaag	2160
	gctcagtcga	aagactgggc	ctttcgtttt	atctgttgtt	tgtcggtgaa	cgctctcctg	2220
	agtaggacaa	atccgcccgg	agcggttttg	aacgttgcca	agcaacggcc	cggaggggtg	2280
25	cgggcaggac	gcccggcata	aactgccagg	catcaaatta	agcagaaggc	cactctgacg	2340
	gatggccttt	ttgcgtttct	acaaactcct	ttgtttattt	ttctaaatac	attcaaatac	2400
	gtatccgctc	atgagacaat	aaccctgata	aatgcttcaa	taataattgaa	aaaggaagag	2460
	tatgagtatt	caacattttc	gtgtcgccct	tattcccttt	tttgcgcat	tttgccctcc	2520
	tgtttttgct	caccagaaa	cgctggtgaa	agtaaaagat	gctgaagatc	agttgggtgc	2580
30	acgagtgggt	tacatcgaac	tggtatctcaa	cagcggttaag	atccttgaga	gttttcgccc	2640
	cgaagaacgt	tttccaatga	tgagcacttt	taaagtctctg	ctatgtggcg	cgggtattatc	2700
	ccgtgttgac	gccgggcaag	agcaactcgg	tcgccgcata	cactattctc	agaatgactt	2760
	ggttgagtac	tcaccagtca	cagaaaagca	tcttacggat	ggcatgacag	taagagaatt	2820
	atgcagtgct	gccataacca	tgagtataaa	cactgcggcc	aacttacttc	tgacaacgat	2880
35	cggaggaccg	aaggagctaa	ccgctttttt	gcacaacatg	ggggatcatg	taactcgcc	2940
	tgatcggttg	gaaccggagc	tgaatgaagc	cataccaaac	gacgagcgtg	acaccacgat	3000
	gacctgtagca	atggcaacaa	cgttgcgcaa	actattaact	ggcgaactac	ttactctagc	3060
	ttccccgcaa	caattaatag	actggatgga	ggcgataaaa	gttgcaggac	cacttctgcg	3120
	ctcgcccttt	ccggctggct	ggtttattgc	tgataaatct	ggagccgggtg	agcggtgggtc	3180
40	tcgcggtatc	attgcagcac	tgggggccaga	tggtaaagccc	tcccgtatcg	tagttatcta	3240
	cacgacgggg	agtcaggcaa	ctatggatga	acgaaataga	cagatcgctg	agataggctg	3300
	ctcactgatt	aagcattggt	aactgtcaga	ccaagtctac	tcatatatac	tttagattga	3360
	tttaaaactt	cttttttaact	ttaaaaggat	ctaggtgaag	atcctttttg	ataatctcat	3420
	gaccaaaatc	ccttaacgtg	agttttcggt	ccaactgagcg	tcagaccccg	tagaaaagat	3480
45	caaaggatct	tcttgagatc	ctttttttct	gcgcgtaatc	tgctgcttgc	aaacaaaaaa	3540
	accaccgcta	ccagcggtgg	tttgtttgcc	ggatcaagag	ctaccaactc	tttttccgaa	3600
	ggtaactggc	ttcagcagag	cgcagatacc	aaatactgtc	cttctagtgt	agccgtagtt	3660
	aggccaccac	ttcaagaact	ctgtagcacc	gcctacatac	ctcgctctgc	taatcctggt	3720
	accagtggct	gctgccagtg	gcgataagtc	gtgtcttacc	gggttggaact	caagacgata	3780
50	gttaccggat	aagggcgagc	ggtcgggctg	aacggggggt	tcgtgcacac	agcccagctt	3840
	ggagcgaag	acctacaccg	aactgagata	cctacagcgt	gagctatgag	aaagcgccac	3900
	gcttcccga	gggagaaaag	cggacaggta	tccggtaaag	ggcagggctg	gaacaggaga	3960
	gcgcacgagg	gagcttccag	ggggaaaacgc	ctggatatctt	tatagtcctg	tcgggtttcg	4020
	ccacctetga	cttgagcgtc	gattttttgtg	atgctcgctca	ggggggcgga	gcctatggaa	4080
55	aaacgcagc	aacggggcct	ttttacgggt	cctggccttt	tgctggcctt	ttgctcacat	4140
	gttcttttcc	gcgttatccc	ctgattcatt	aatgcaggtc	acgatacctt	ctggcgagtc	4200
	cccgtgcgga	gtcggagagc	gctccctgag	cgcgtgcggc	ccgagaggtc	gcgcctggcc	4260
	ggccttcggt	ccctcgtgtg	tcccgtcggt	aggaggggcc	ggccgaaaat	gcttccggct	4320
	cccgcctctg	agacacgggc	cggccccctg	cgtgtggcac	gggcggccgg	gagggcgctcc	4380
60	ccggccccgg	gctgtctccg	cgtgtgtcct	ggggttgacc	agagggcccc	gggcgctccg	4440
	tgtgtggctg	cgatggtggc	gtttttgggg	acaggtgtcc	gtgtccgtgt	cgcgcgctcg	4500

ctgggcccggc ggcgtgggtcg gtgacgcgac ctcccggccc cgggggaggt atatctttcg 4560  
ctccgagtcg gcattttggg ccgcccgggt attagtagaa acaggggtac 4610

5 <210> 7  
<211> 3558  
<212> DNA  
<213> Artificial Sequence

10 <220>  
<223> Description of Artificial Sequence: pHL2583

<400> 7  
tattagtaga aacaggggtat tttttattct agtacattac gccccgccct gccactcacc 60  
15 gcagtactgt tgtaattcat taagcattct gccgacatgg aagccatcac agacggcatg 120  
atgaacctga atcgccagcg gcatcagcac cttgtcgccct tgcgtataat atttgcccat 180  
gggtgaaaacg ggggcgaaga agttgtccat attggccacg tttaaatcaa aactgggtgaa 240  
actcaccacg ggattggctg agacgaaaaa catattctca ataaaccctt tagggaaata 300  
ggccagggttt tcaccgtaac acgccacatc ttgcgaatat atgtgtagaa actgccggaa 360  
20 atcgtcgtgg tattcactcc agagcgtatg aaacgtttca gtttgctcat ggaaaacggg 420  
gtaacaaggg tgaacactat cccatatcac cagctcaccg tctttcattg ccatacggaa 480  
ttccggatga gcattctca ggccggcaag aatgtgaata aaggccggat aaaacttgtg 540  
cttatttttc tttacggtct ttaaaaaggc cgtaatatcc agctgaacgg tctgggtata 600  
ggtacattga gcaactgact gaaatgcctc aaaatgttct ttacgatgcc attgggatat 660  
25 atcaacgggtg gtatatccag tgattttttt ctccatgatt atggccatta cccttgtttc 720  
tactcccccc caacttcgga ggtcgaccag tactccgggc gaaactttgt tttttttttt 780  
tcccccgatg ctggagggtcg accagatgtc cgaaagtgtc cccccccccc cccccccccc 840  
gcgcgggaacg gcggggccac tctggactct tttttttttt tttttttttt tttttgggga 900  
tcggcccgctg gcttctgttt tggcggatga gagaagattt tcagcctgat acagattaaa 960  
30 tcagaacgca gaagcgggtct gataaaacag aatttgccctg gcggcagtag cgcggtggtc 1020  
ccacctgacc ccatgccgaa ctcagaagtg aaacgccgta gcgcgatgg tagtggtggg 1080  
tctccccatg cgagagtagg gaactgccag gcatcaaata aaacgaaagg ctcatcgaa 1140  
agactgggccc tttcggtttta tctgttgttt gtcggtgaac gctctcctga gtaggacaaa 1200  
tccgcgggga gcggatttga acgttgcgaa gcaacggccc ggagggtggc ggccaggacg 1260  
35 cccgccataa actgccaggc atcaaattaa gcagaaggcc atcctgacgg atggcctttt 1320  
tgcggtttcta caaactcttt tgtttatttt tctaaataca ttcaaataatg tatccgctca 1380  
tgagacaata accctgataa atgcttcaat aatattgaaa aaggaaaggt atgagtattc 1440  
aacatttccg tgtcgccctt attccctttt ttgcggcatt ttgccttctt gtttttgctc 1500  
acccagaaac gctggtgaaa gtaaaagatg ctgaagatca gttgggtgca cgagtgggtt 1560  
40 acatcgaact ggatctcaac agcggtaaga tccttgagag ttttcgcccc gaagaacgtt 1620  
ttccaatgat gagcactttt aaagtctctg actattctca gaatgacttg gttgagtact 1740  
ccgggcaaga gcaactcggg cgcgcgcatc actatctcta aagagaatta tgcagtgtct 1800  
cccaactcac agaaaagcat cttacggatg gcatgacagt aagagaatta tgcagtgtct 1860  
45 ccataaccat gagtgataac actgcggcca acttacttct gacaacgacg ggaggaccga 1920  
aggagctaac cgcttttttg cacaacatgg gggatcatgt aactcgcctt gatcgttggg 1980  
aaccggagct gaatgaagcc ataccaaacg acgagcgtga caccacgatg cctgtagcaa 2040  
tggcaacaac gttgcgcaaa ctattaactg gcgaactact tactctagct tcccggaac 2100  
aattaataga ctggatggag gcggataaag ttgcaggacc acttctgcgc tcggcccttc 2160  
cggtctggctg gtttattgct gataaatctg gagccggtga gcgtgggtct cgcggtatca 2220  
50 ttgcagcact ggggccagat ggtaagccct cccgtatcgt agttatctac acgacgggga 2280  
gtcaggcaac tatggatgaa cgaaatagac agatcgctga gataggtgcc tcaactgatta 2340  
agcattggta actgtcagac caagtttact catatatact ttagattgat ttaaaacttc 2400  
atttttaatt taaaaggatc taggtgaaga tcctttttga taatctcatg accaaaatcc 2460  
cttaacgtga gttttcgttc cactgagcgt cagaccccggt agaaaagatc aaaggatctt 2520  
55 cttgagatcc tttttttctg cgcgtaatct gctgcttgca aacaaaaaaa ccaccgctac 2580  
cagcgggtgg ttgtttgccc gatcaagagc taccgaactct ttttcggaag gtaactggct 2640  
tcagcagagc gcagatacca aatactgtcc ttctagtga gccgtagtta ggccaccact 2700  
tcaagaactc tgtagcaacc cctacatacc tcgctctgct aatcctgtta ccagtggctg 2760  
ctgccagtgc gataaagtcg tgtcttaccg ggttggactc aagacgatag ttaccggata 2820  
60 aggcgcagcg gtcgggctga acgggggggt cgtgcacaca gccagcttg gagcgaacga 2880  
cctacaccga actgagatac ctacagcgtg agctatgaga aagcgcacg cttcccgaag



ggagaaaggc ggacaggtat ccggtaaagcg gcagggtcgg aacaggagag cgcacgaggg 2940  
 agcttccagg gggaaacgcc tggatatctt atagtcctgt cgggtttcgc cacctctgac 3000  
 ttgagcgtcg atttttgtga tgctcgtcag gggggcggag cctatggaaa aacgccagca 3060  
 acgcggcctt tttacgggtc ctggcctttt gctggccttt tgctcaccatg ttctttcctg 3120  
 5 cgttatcccc tgattcatta atgcagggtca cgatcctttc tggcgagtcc ccgtgcggag 3180  
 tcggagagcg ctccctgagc gcgtgcggcc cgagaggtcg cgcctggccg gccttcggtc 3240  
 cctcgtgtgt cccggtcgta ggagggggccg gccgaaaatg ctcccggtc ccgctctgga 3300  
 gacacgggccc ggccccctgc gtgtggcacg ggcgccggg agggcgctccc cggccccggcg 3360  
 ctgctccccg gtgtgtcctg gggttgacca gagggccccg ggcgctccgt gtgtggctgc 3420  
 10 gatggtggcg tttttgggga caggtgtccg tgtccgtgtc gcgcgtcgcc tgggcccggcg 3480  
 gcgtggtcgg tgacgcgacc tcccggcccc gggggaggtat tatctttcgc tccgagtcgg 3540  
 cattttgggc cgccgggt 3558

15 <210> 8  
 <211> 4343  
 <212> DNA  
 <213> Artificial Sequence

20 <220>  
 <223> Description of Artificial Sequence: pHL2989

<400> 8  
 25 ctttctggcg agtccccgtg cggagtcgga gagcgctccc tgagcgcgtg cggccccgaga 60  
 ggtcgcgcct ggccggcctt cggtcctcgt tgtgtcccg tctagtaggagg ggccggccga 120  
 aaatgcttcc ggctcccgtc ctggagacac gggccggccc cctgcgtgtg gcacggggcg 180  
 ccgggagggc gtccccggcc cggcgctgct cccgcgtgtg tcttgggggt gaccagaggg 240  
 ccccgggcgc tccgtgtgtg gctgcgatgg tggcgctttt ggggacaggt gtccgtgtcc 300  
 gtgtcgcgcg tgcctggggc cggcggcgtg gtcgggtgacg cgacctcccg gccccggggg 360  
 30 aggtatatct ttcgctccga gtcggcattt tgggcccggc gggtattagt agaaacaggg 420  
 tattttttat actagtaagc tcgaaggagt ccaccatgag taaaggagaa gaacttttca 480  
 ctggagttgt cccaattcct gttgaattag atggtgatgt taatgggcac aaattttctg 540  
 tcaagtggaa ggggtgaagg gatgcaacat acggaaaact tacccttaaa ttattttgca 600  
 ctactggaaa actacctgtt ccattgtcca cactttcact tatggtgttc 660  
 35 aatgcttttc aagataccca gatcatatga aacagcatga ctttttcaag agtgccatgc 720  
 ccgaagggtta tgtacaggaa agaactatat ttttcaaaga tgacgggaac tacaagacac 780  
 gtgctgaagt caagtgtgaa ggtgataccc ttgttaatag aatcgagtta aaaggtattg 840  
 atttttaaaga agatggaaac attcttgga acaaattgga atacaactat aactcacaca 900  
 atgtatacat catggctgac aagcagaaga acggaatcaa ggccaacttc aagacccgcc 960  
 40 acaacatcga ggacggcgcc gtgcagctgg ccgaccata ccagcagaac accccaattg 1020  
 gcatgagccc gtctcttta ccagacaacc attactgtc cacacaactc gccctttcga 1080  
 aagatcccaa gtaaaagaga gaccacatgg tcttcttga gtttgtaaca gctgctggga 1140  
 ttacacatgg catggatgaa ctatacaagg gatcccatca ccatcaccat cactaagctc 1200  
 catggtctag atatctagta cattacgccc cgccctgcca ctcatcgag tactgttgta 1260  
 45 attcattaag cattctgccg acatggaagc catcacagac ggcagatga acctgaatcg 1320  
 ccagcggcat cagcaccttg tgccttgcg tataatattt gcccatggtg aaaacggggg 1380  
 cgaagaagtt gtccatattg gccacgttta aatcaaaact ggtgaaactc acccagggat 1440  
 tggcactcac aaagaacatg ttctcgatga atcctttagg gaagtaggcc aggttttcac 1500  
 cgtaaacacgc cacatcttgc gaatatatgt gttagaaactg ccggaatcg tctggttatt 1560  
 50 cactccagag cgatgaaaac gtttcagttt gctcatggaa aacggtgtaa caagggtgaa 1620  
 cactatccca taccacgagc tcaccgtctt tcattgccat acggaattcc ggatgagcat 1680  
 tcatcaggcg ggcaagaatg tgaataaagg ccggataaaa cttgtgctta ttttcttta 1740  
 cggctcttta aaaggccgta atatccagct gaacggtctg gttataggta cattgagcaa 1800  
 ctgactgaaa tgcctcaaaa tgttctttac gatgccattg ggatatatca acggtggtat 1860  
 55 atccagtgat tttttcttcc atgattatgc aaaaaatacc cttgtttcta cccccccca 1920  
 acttcggagg tgcaccagta ctcggggcga aactttgttt ttttttttc ccccgatgct 1980  
 ggaggtcgac cagatgtccg aaagtgtccc cccccccccc cccccccggc gcggaacggc 2040  
 gggggcactc tggactcttt tttttttttt tttttttttt tttggggatc ggccgctagc 2100  
 ttctgttttg gcggatgaga gaagattttc agcctgatac agattaaatc agaacgcaga 2160  
 60 agcggctctga taaaacagaa tttgcctggc ggcagtagcg cgggtggtccc acctgacccc 2220  
 atgccgaact cagaagtgaac acgccgtagc gccgatggta gtgtggggtc tccccatgcy 2280



```

5  agagtagggga actgccaggc atcaaataaa acgaaaggct cagtcgaaaag actggggcctt 2340
   tcgtttttatc tgttgtttgt cgggtgaacgc tctcctgagt aggacaaatc cgccgggagc 2400
   ggatttgaac gttgcgaagc aacggcccgg aggggtggcgg gcaggacgcc cgccataaac 2460
   tgccaggcat caaatthaagc agaaggccat cctgacggat ggcctttttg cgtttctaca 2520
   aactcctttt tttatttttc taaatacatt caaatatgta tccgctcatg agacaataac 2580
   cctgataaat gcttcaataa tattgaaaaa ggaagagtat gagtattcaa catttccgtg 2640
   tcgcccttat tccctttttt gcggcatttt gccttcctgt ttttgctcac ccagaaacgc 2700
   tgggtgaaagt aaaagatgct gaagatcagt tgggtgcacg agtgggttac atcgaactgg 2760
   atctcaacag cggtaagatc cttgagagtt ttcgccccga agaacgtttt ccaatgatga 2820
10  gcacttttaa agttctgcta tgtggcgcgg tattatcccg tgttgacgcc gggcaagagc 2880
   aactcggtcg ccgcatacac tattctcaga atgacttggt tgagtactca ccagtcacag 2940
   aaaagcatct tacggatggc atgacagtaa gagaattatg cagtgtctgc ataaccatga 3000
   gtgataaacac tgcggccaac ttacttctga caacgatcgg aggaccgaag gagaccgaag 3060
   cttttttgca caacatgggg gatcatgtaa ctgccttga tctgtgggaa ccggagctga 3120
15  atgaagccat accaaacgac gacgctgaca ccacgatgcc tgtagcaatg gcaacaacgt 3180
   tgcgcaaac atttaactggc gaactactta ctctagcttc ccggcaacaa ttaatagact 3240
   ggatggaggc ggataaagtt gcaggaccac ttctgcgctc ggcccttccg gctggctgg 3300
   ttattgctga taaatctgga gccggtgagc gtgggtctcg cggtatcatt gcagcactgg 3360
   ggccagatgg taagccctcc cgtatcgtag ttatctacac gacggggagt caggcaacta 3420
20  tggatgaacg aaatagacag atcgctgaga taggtgcctc actgattaag cattggaac 3480
   tgtcagacca agtttactca tatatacttt agattgattt aaaacttcat ttttaattta 3540
   aaaggatcta ggtgaagatc ctttttgata atctcatgac caaaatccct taacgtgagt 3600
   tttcgttcca ctgagctgca gaccccgtag aaaagatcaa aggatcttct tgagatcctt 3660
   tttttctgcg cgtaatctgc tgcttgcaaa caaaaaaacc accgctacca gcggtgggtt 3720
25  gtttgccgga tcaagagcta ccaactcttt ttccgaaggt aactggcttc agcagagcgc 3780
   agataccaaa tactgtcctt ctagtgtagc cgtagttagg ccaccacttc aagaactctg 3840
   tagcaccgcc tacatacctc gctctgctaa tcctgttacc agtggctgct gccagtgccg 3900
   ataagtcgtg tcttaccggg ttggactcaa gacgatagtt accggataag gcgcagcgg 3960
   cgggctgaac ggggggttcg tgcacacagc ccagcttgga gcgaacgacc tacaccgaac 4020
30  tgagataacct acagcgtgag ctatgagaaa gcgccacgct tcccgaaggg agaaaggcgg 4080
   acaggtatcc ggtaagcggc agggctcgga caggagagcg cacgagggag cttccagggg 4140
   gaaacgcctg gtatctttat agtcctgtcg ggtttcgcca cctctgactt gagcgtcgat 4200
   ttttgtgatg ctcgtcaggg gggcggagcc tatggaaaaa cgccagcaac gcggcctttt 4260
   tacggttcct ggcccttttg tggccttttg ctcacatggt ctttctctgc ttatccctg 4320
35  attcattaat gcaggtcacg atc 4343

```

&lt;210&gt; 9

&lt;211&gt; 3888

40 &lt;212&gt; DNA

&lt;213&gt; Artificial Sequence

&lt;220&gt;

&lt;223&gt; Description of Artificial Sequence: pHL1920

```

45  <400> 9
   cccaaaaaaa aaaaaaaaaa aaaaaaaaaa agtccagagt ggccccgccg ttccgcgccg 60
   gggggggggg ggggggggga cactttcgga catctggtcg acctccagca tcgggggaaa 120
   aaaaaaaaac aaagtttcgc ccggagtact ggtcgacctc cgaagtggg ggggagtaga 180
50  aacagggtag ataatcactc actgagtgc atccacatcg cgagcgcgcg taatacgact 240
   cactataggg cgaattgggt accgggcccc cctcgaggt cgacggtatc gataagcttc 300
   gacgagattt tcaggagcta aggaagctaa aatggagaaa aaaatcactg gatataccac 360
   cgttgatata tcccaatggc atcgtaaaga acattttgag gcatttcagt cagtgtctca 420
   atgtacctat aaccagaccg ttcagctgga tattacggcc tttttaaaga ccgtaaagaa 480
55  aaataagcac aagtttttat cggcctttat tcacattctt gcccgctga tgaatgctca 540
   tccggaattc cgtatggcaa tgaaagacgg tgagctgggtg atatgggata gtgttcaccc 600
   ttgttacacc gttttccatg agcaaaactga aacgthttca tcgctctgga gtgaatacca 660
   cgacgatttc cggcagtttc tacacatata ttcgcaagat gtggcggtgt acggtgaaaa 720
   cctggcctat ttccctaaag ggtttattga gaatatgttt ttcgtctcag ccaatccctg 780
60  ggtgagtttc accagttttg atttaaacgt ggccaatatg gacaacttct tcgcccccg 840
   tttcaccatg ggcaaatatt atacgcaagg cgacaagggt ctgatgccgc tggcgattca 900

```

5 gggtcatcat gccgtttgtg atggcttcca tgtcggcaga atgcttaatg aattacaaca 960  
 gtactgcat gagtggcagg gcggggcgta atttttttaa ggcagttatt ggtgccctta 1020  
 aacgcctggt gctacgcctg aataagtgat aataagcgga tgaatggcag aaattcgtcg 1080  
 aagcttgata tcgaattcct gcagcccggt ggatccacta gttctagagc ggccgccacc 1140  
 gcggtggagc tccagctttt gttcccttta gtgagggtta attgcgcgca ggcctagcta 1200  
 ggtaaaagaaa aatacccttg attcttctaa taaccgcggt gcccaaaatg ccgactcgga 1260  
 gcgaaagata tacctcccc gggtccggga ggtcgcgtca ccgaccacgc cgcgcgcca 1320  
 ggcgacgcgc gacacggaca cctgtcccca aaaacgccac catcgagcc acacacggag 1380  
 10 cgcccggggc cctctgttca accccaggac acacgcggga gcagcgccgg gccggggagc 1440  
 ccctcccggc cgcccggtgc acacgcaggg ggccggcccg tgtctccaga gcgggagccg 1500  
 gaagcatttt cggtccggcc ctctacgcag cgggacacac gagggaccga aggcggcca 1560  
 ggcgcgaccc tccgggcgc acgcgcgtc agggagcgt ctccgactcc gcacggggac 1620  
 tcgccagaaa ggatcgtgac ctgcattaat gaatcagggg ataacgcagg aaagaacatg 1680  
 tgagcaaaaag gccagcaaaa ggccaggaac cgtaaaaagg ccgcgttgct ggcgtttttc 1740  
 15 cataggctcc gccccctga cgagcatcac aaaaatcgac gctcaagtca gaggtggcga 1800  
 aaccgcacag gactataaag ataccaggcg ttccccctg gaagctccct cgtgcgtctt 1860  
 cctgttccga ccctgccgct taccggatc ctgtccgct ttctcccttc gggaaagcgtg 1920  
 gcgctttctc atagctcacg ctgtaggat ctcagttcgg tgtaggtcgt tcgctccaag 1980  
 ctgggtgtgt tgcacgaacc cccgttcag cccgaccgt gcgccttatc cggtaactat 2040  
 20 cgtcttgagt ccaaccgggt aagacacgac ttatcgccac tggcagcagc cactggtaac 2100  
 aggattagca gagcgaggta tgtaggcggt gctacagagt tcttgaagtg gtggcctaac 2160  
 tacggctaca ctagaaggac agtatttgg atctgcgtc tgctgaagcc agttaccttc 2220  
 ggaaaaagag ttggtagctc ttgatccggc aaacaaacca ccgctggtag cgggtggtttt 2280  
 tttgtttgca agcagcagat tacgcgcaga aaaaaaggat ctcaagaaga tcctttgatc 2340  
 25 ttttctacgg ggtctgacgc tcagtggaa gaaaactcac gttaaggat tttgggtcatg 2400  
 agattatcaa aaaggatctt cacctagatc cttttaaat aaaaatgaag ttttaactca 2460  
 atctaaagta tatatgagta aacttggtct gacagttacc aatgcttaat cagtgaggca 2520  
 cctatctcag cgatctgtct atttcgttca tccatagtgt cctgactccc cgtcgtgtag 2580  
 ataactacga tacgggaggg cttaccatct ggccccagt ctgcaatgat accgcgagac 2640  
 30 ccacgctcac cggctccaga tttatcagca ataaaccagc cagccggaag ggccgagcgc 2700  
 agaagtggtc ctgcaacttt atccgcctcc atccagtcta ttaattgttg ccgggaagct 2760  
 agagtaagta gttcgccagt taatagtttg cgcaacgttg ttgccattgc tacaggcatc 2820  
 gtggtgtcac gctcgtcgtt tggatggct tcattcagct ccggttccca acgatcaagg 2880  
 cgagttacat gatcccccat gttgtgcaaa aaagcggtta gctccttcgg tccctcgatc 2940  
 35 gttgtcagaa gtaagtgtgc cgcagtgtta tcaactcatg ttatggcagc actgcataat 3000  
 tctcttactg tcatgccatc cgtaagatgc ttttctgtga ctggtgagta ctcaaccaag 3060  
 tcattctgag aatagtgtat gcggcgaccg agttgctctt gcccggcgtc aacacgggat 3120  
 aataccgcgc cacatagcag aactttaaaa gtgctcatca ttggaaaacg ttcttcgggg 3180  
 cgaaaactct caaggatctt accgctgttg agatccagtt cgatgtaacc cactcgtgca 3240  
 40 cccaactgat cttcagcatc ttttactttc accagcgttt ctgggtgagc aaaaacagga 3300  
 aggcataaat cgcaaaaaa gggaataagg gcgacacgga aatgttgaat actcatactc 3360  
 ttcctttttc aatattattg aagcatttat cagggttatt gtctcatgag cggatacata 3420  
 tttgaaatgta tttagaaaaa taaacaaaag agtttgtaga aacgcataaa ggccatccgt 3480  
 caggatggcc ttctgcttaa tttgatgcct ggcagtttat ggccggcgct ctgcccgcga 3540  
 45 ccctccgggc cgttgcttcg caacgttcaa atccgctccc ggccgatttg tccactcag 3600  
 gagagcgttc accgacaaac aacagataaa acgaaaggcc cagtctttcg actgagcctt 3660  
 tcgttttatt tgatgcctgg cagttcccta ctctcgcagt gggagacccc aactaccatc 3720  
 cggcgctacg gcgtttcact tctgagttcg gcatggggtc aggtgggacc accgcgctac 3780  
 tgccgcaggg caaattctgt tttatcagac cgcttctgcg ttctgattta atctgtatca 3840  
 50 ggctgaaaat cttctctcat ccgccaaaac agaagctagc ggccgac 3888

&lt;210&gt; 10

&lt;211&gt; 12

55 &lt;212&gt; RNA

&lt;213&gt; Influenza A virus

&lt;400&gt; 10

ccugcuuuug cu

12

60

5 <210> 11  
<211> 12  
<212> RNA  
<213> Influenza B virus  
  
<400> 11  
nnygcuucug cu 12

10 <210> 12  
<211> 12  
<212> RNA  
<213> Influenza C virus

15 <400> 12  
ccugcuucug cu 12

20 <210> 13  
<211> 12  
<212> RNA  
<213> Artificial Sequence

25 <220>  
<223> Description of Artificial Sequence: Modified  
influenza A 3' sequence (pHL1104 and 1920)

30 <400> 13  
ccuguuucua cu 12

35 <210> 14  
<211> 12  
<212> RNA  
<213> Artificial Sequence

40 <220>  
<223> Description of Artificial Sequence: Modified  
influenza A 3' sequence (pHL1948)

<400> 14  
ccucguucuc cu 12

45 <210> 15  
<211> 13  
<212> RNA  
<213> Artificial Sequence

50 <220>  
<223> Description of Artificial Sequence: Modified  
influenza A 5' sequence (pHL1920)

55 <400> 15  
agaagaaucagg 13

60 <210> 16  
<211> 13  
<212> RNA  
<213> Influenza A virus

<400> 16  
aguagaaaca agg 13

5  
<210> 17  
<211> 13  
<212> RNA  
<213> Influenza B virus

10  
<400> 17  
aguagwaaca rnn 13

15  
<210> 18  
<211> 13  
<212> RNA  
<213> Influenza C virus

20  
<400> 18  
agcaguagca agr 13

25  
<210> 19  
<211> 21  
<212> RNA  
<213> Influenza A virus

30  
<400> 19  
aguagaaaca aggnnnuuuu u 21

35  
<210> 20  
<211> 21  
<212> RNA  
<213> Artificial Sequence

40  
<220>  
<223> Description of Artificial Sequence: Modified  
influenza A 5'-sequence (pHL1920)

<400> 20  
agaagaauca aggnnnuuuu u 21

45  
<210> 21  
<211> 21  
<212> RNA  
<213> Influenza B virus

50  
<400> 21  
aguagwaaca rnnnnuuuu u 21

55  
<210> 22  
<211> 19  
<212> RNA  
<213> Artificial Sequence

60  
<220>  
<223> Description of Artificial Sequence: Modified

## influenza C 5' sequence

5 <400> 22  
aguaguaaca agrguuuuu 19

10 <210> 23  
<211> 15  
<212> RNA  
<213> Artificial Sequence

15 <220>  
<223> Description of Artificial Sequence: Modified  
influenza A 3' sequence (pHL1104 and 1920)

<400> 23  
nnnccucuuu cuacu 15

20 <210> 24  
<211> 15  
<212> RNA  
<213> Artificial Sequence

25 <220>  
<223> Description of Artificial Sequence: Modified  
influenza A 3' sequence (pHL1948)

30 <400> 24  
nnnccucguu cuccu 15

35 <210> 25  
<211> 15  
<212> RNA  
<213> Artificial Sequence

40 <220>  
<223> Description of Artificial Sequence: Modified  
influenza B 3' sequence

<400> 25  
nnnnnyguuu cuacu 15

45 <210> 26  
<211> 14  
<212> RNA  
<213> Artificial Sequence

50 <220>  
<223> Description of Artificial Sequence: Modified  
influenza C 3' sequence

55 <400> 26  
ccccuguuuc uacu 14

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/01903

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 7 C12N15/86 C12N7/01 C12N5/10 A61K39/00 A61K39/145  
A61K48/00

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 7 C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DE 197 09 512 A (HOBOM GERD PROF DR DR) 10 September 1998 (1998-09-10)  the whole document ---	1,4,5, 12,14, 18-33
Y	WO 91 03552 A (SINAI SCHOOL MEDICINE) 21 March 1991 (1991-03-21)  figure 11; example 7 ---	1,4,5, 12,14, 18-33
Y	TAKASE H. ET AL: "Antibody responses and protection in mice immunized orally against influenza virus." VACCINE, vol. 14, no. 17/18, 1996, pages 1651-1656, XP002110225 page 1652, left-hand column, paragraph 1 ---	27
	--- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

### \* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

15 June 2000

Date of mailing of the international search report

07/07/2000

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl.  
Fax: (+31-70) 340-3016

Authorized officer

Mandl, B

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/01903

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	ZHOU Y. ET AL.: "Membrane-anchored incorporation of a foreign protein in recombinant Influenza virions." VIROLOGY, vol. 246, 20 June 1998 (1998-06-20), pages 83-94, XP002110226 the whole document ---	5
A	ZOBEL A. ET AL.: "RNA polymerase I catalysed transcription of insert viral cDNA." NUCLEIC ACIDS RESEARCH, vol. 21, no. 16, 1993, pages 3607-3614, XP002110227 page 3607, right-hand column, paragraph 2 page 3612, right-hand column, paragraph 2 page 3613, left-hand column, line 1 page 3614, left-hand column, paragraph 2 ---	15-17
A	WO 96 10641 A (BAYER AG ;HOBOM GERD (DE); NEUMANN GABRIELE (DE); MENKE ANNETTE (D) 11 April 1996 (1996-04-11) cited in the application the whole document ---	6-11
A	FLICK R. ET AL.: "Promoter elements in the influenza vRNA terminal structure." RNA, vol. 2, no. 10, 1996, pages 1046-1057, XP000914725 ISSN: 1355-8382 the whole document ---	6-11
A	NEUMANN G. AND HOBOM G.: "Mutational analysis of influenza virus promoter elements in vivo." JOURNAL OF GENERAL VIROLOGY 1995, vol. 76, no. 7, 1995, pages 1709-1717, XP002140118 ISSN: 0022-1317 cited in the application ---	6-11
A	PICCONE M. E. ET AL.: "MUTATIONAL ANALYSIS OF THE INFLUENZA VIRUS vRNA PROMOTER" VIRUS RESEARCH, vol. 28, no. 2, 1 January 1993 (1993-01-01), pages 99-112, XP000619019 ISSN: 0168-1702 the whole document ---	6-11

-/--

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/EP 00/01903

## C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	PALESE P. ET AL.: "Negative-strand RNA viruses: Genetic engineering and applications." PROC. NATL. ACAD. SCI. U.S.A., vol. 93, October 1996 (1996-10), pages 11354-11358, XP000196755 page 11354, right-hand column, last paragraph -page 11356, right-hand column, paragraph F ---	5
P,X	NEUMANN G. ET AL.: "Plasmid-driven formation of influenza virus-like particles." JOURNAL OF VIROLOGY, vol. 74, no. 1, January 2000 (2000-01), pages 547-551, XP002140119 ISSN: 0022-538X the whole document ---	1,3-5, 12, 18-23, 25-31,33
P,A	FLICK R. AND HOBOM G.: "Interaction of influenza virus polymerase with viral RNA in the 'corkscrew' conformation." JOURNAL OF GENERAL VIROLOGY, vol. 80, no. 10, October 1999 (1999-10), pages 2565-2572, XP002140120 ISSN: 0022-1317 figure 1 -----	7-11



# INTERNATIONAL SEARCH REPORT

Information on patent family members

Int. Application No

PCT/EP 00/01903

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
DE 19709512 A	10-09-1998	NONE	
WO 9103552 A	21-03-1991	US 5166057 A AT 126272 T AU 636916 B AU 6411890 A CA 2065245 A DE 69021575 D DE 69021575 T DK 490972 T EP 0490972 A ES 2075901 T GR 90100639 A JP 5500607 T PT 95124 A US 5252289 A US 6001634 A US 5578473 A US 5854037 A US 5840520 A US 5786199 A US 5820871 A ZA 9006852 A	24-11-1992 15-08-1995 13-05-1993 08-04-1991 01-03-1991 14-09-1995 14-12-1995 30-10-1995 24-06-1992 16-10-1995 30-12-1991 12-02-1993 18-04-1991 12-10-1993 14-12-1999 26-11-1996 29-12-1998 24-11-1998 28-07-1998 13-10-1998 31-07-1991
WO 9610641 A	11-04-1996	EP 0704533 A AU 3607695 A EP 0783586 A FI 971272 A NZ 293600 A	03-04-1996 26-04-1996 16-07-1997 26-05-1997 28-01-1999

**THIS PAGE BLANK (USPTO)**